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Reproduction in diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae): morphology, behavior, and chemical ecology

by

Kristine Ann Justus

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Entomology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "**Reproduction in diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae): morphology, behavior, and chemical ecology**" submitted by Kristine Ann Justus in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Reproduction by *Plutella xylostella* (L.), the diamondback moth, was investigated in a series of experiments employing several techniques to ascertain details of copulation and oviposition. Internal reproductive morphology and genitalic configurations during copulation were elucidated. Reproductive structures were mostly typical of Lepidoptera with a few peculiarities. Morphometric differences were observed in comparisons of seven distantly-separated populations, and the possibility that larval diets effect these differences is discussed. Observations of ovipositing moths led to descriptions of behaviors that occur in probabilistic sequence; these behaviors are thought to facilitate identification of potential oviposition sites. Bioassays suggested contact chemical stimuli are required for egg deposition but plant volatiles decrease time required for host acceptance. Tactile information appeared to be important for egg location only. Field and laboratory choice experiments with normal (waxy) and reduced (glossy) wax blooms provided evidence that epicuticular features are extremely important in host preferences. Glossy plants accumulated more eggs regardless of plant age. However, similar numbers of eggs were deposited on parafilm-transfers of leaf surfaces and characteristics other than parafilm adherents must be involved in distinguishing glossy from waxy morphotypes. Headspace extracts of glossy and waxy *B. napus*, *S. alba*, and *D. carota* were collected and used in gas-chromatography coupled with electroantennography. Females and males responded to several components of *B. napus* and *S. alba* extracts, but components eliciting responses did not necessarily co-occur. Few responses were elicited by volatiles of *D. carota*, a non-host plant. It is likely that individual components of plant volatiles combine to form signature blends and act as an initial diagnostic character

of host plants. Additionally, electrophysiology of contact chemosensory sensilla revealed at least two types of contact sensilla (those that respond to KCl and those that do not) and the existence of a polar compound on the surface of *B. napus* and *S. alba* that is detected by gustatory sensilla of the antennae. Possible plant characteristics influencing reproduction by *P. xylostella* are discussed.

For Angel

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List of Symbols and Abbreviations

+	< 20% responding
++	approximately 50% responding
+++	> 80% responding
*	poor response
**	fair response
***	good response
****	excellent response
✓	selected protocol
Å	angstroms (10^{-10} meters)
°C	degrees Celsius
Ω	ohm
µl	microliters
µm	micrometers
ac	accessory gland
acf	activated charcoal filter
ad	aedeagus
Ag-AgCl	chloridized silver
an	anterior
AN	antennation
AR	antennal rotation
asv	accessory seminal vesicle
bal	ballast tank
bas	plant basin
bc	bursa copulatrix
bd	bursal duct
bs	bud-shaped sensillum
car	*Daucus carota*
cbs	complete band of scales
cg	colleterial gland

ch	chaetic sensillum
CIF	cabbage identification factor
cl	coeloconic sensillum
cm	centimeters
CNS	central nervous system
Comb100KCl	0.625 gle *B. napus* and *S. alba* extracts (50:50) plus 100 mM KCl
cs	cuticular simplex
ct	cuticle
cv	scale cavity
d	dorsal
DC	direct current
di	dilation
diam	diameter
EAG	electroantennography, electrantennogram
edd	ejaculatory duct duplex
eds	ejaculatory duct simplex
EI	electron impact
EPTC	*S*-ethyl dipropylthiocarbamate
eV	electronvolts
f	cuticular finger
fb	fatbody
fgls	field-grown glossy *B. napus*
FID	flame ionization detector
fwax	field-grown waxy *B. napus*
g	grams
GC	gas chromatography
GC-EAG	gas chromatography coupled with electroantennography
GC-MS	gas chromatography coupled with mass spectrometry
ggls	glasshouse-grown glossy *B. napus*
gle	gram leaf equivalent
gls	glossy *B. napus*

GR	grooming
GRV	grooves
gwf	gaswash flask
gwax	glasshouse-grown waxy *B. napus*
H2O	deionized water
HIA	high input impedance preamplifier
hp	hair pencils
HPF	high pass filter
Hz	hertz
id	inner diameter
IE	indifferent electrode
is	intersegmental membrane
KCl	potassium chloride
kg	kilograms
KOH	potassium hydroxide
la	long aporous sensillum
LEAR	low erucic acid rape
l/min	liters per minute
LPF	low pass filter
lu	lumen
m	meters
min	minutes
mm	millimeters
msec	milliseconds
mt	Malpighian tubule
mtr	flow meter
mu	muscle
mV	millivolts
m/z	mass-to-charge ratio
n	nucleus
NAP	*B. napus* cv. Westar squash

nm	response not measurable
nr	no response
ns	not significant
od	oviduct
or	ovariole
OS	ovipositor sweep
OS^{sr}	ovipositor sweep, search phase
OS^{dt}	ovipositor sweep, determinant phase
OV	oviposition
pbs	partial band of scales
pd	pedicel
PE	proboscis extension
prep	preparation
ps	posterior
pt	protuberance
RE	recording electrode
s	cuticular spine
sa	short aporous sensillum
sc	scale
sd	seminal duct
sec	seconds
sin	*S. alba*
SIN	0.5 mM sinigrin
sk	socket
sp	spermatheca
spg	spermathecal gland
st	styloconic sensillum
ST	still
tf	terminal filament
trI	trichodeal sensillum type I
trII	trichodeal sensillum type II

ts	testes
TTL	total
v	ventral
vac	vacuum
vd	vas deferens
VOL	*B. napus* cv. Westar volatiles
wax	waxy *B. napus*
0.625Bn0KCl	0.625 gle *B. napus* extract
0.625Bn100KCl	0.625 gle *Brassica napus* extract plus 100 mM KCl
0.625Bn500KCl	0.625 gle *B. napus* extract plus 500 mM KCl
0.625Sa0KCl	0.625 gle *Sinapis alba* extract plus 500 mM KCl
0.625Sa100KCl	0.625 gle *S. alba* extract
0.625Sa500KCl	0.625 gle *S. alba* extract plus 500 mM KCl
1.25Bn100KCl	1.25 gle *B. napus* extract plus 100 mM KCl
2.5Bn100KCl	2.5 gle *B. napus* extract plus 100 mM KCl

Chapter 1:

General Introduction

1.1 Cruciferae and secondary compounds

Cruciferae are dicotyledonous herbs (order Capparales), characterized by hypogynous, regular, cruciform flowers. *Brassica* is one of 350 genera of known crucifers (Röbbelen and Downey, 1989) and *Brassica* spp. are economically important agricultural crops: root vegetables (*e.g.* turnip), cole crops (*e.g.* cabbage and broccoli), and oilseeds (*e.g.* rape and mustard).

Rapeseed (*B. napus* and *B. rapa*) has been grown in Canada since 1942 and, by 1966, was the fourth largest of Canada's field crops in terms of hectares seeded (anonymous, 1981). At that time, agricultural varieties of rapeseed had high levels of erucic acid and glucosinolates. Erucic acid comprises up to 40% of fatty acids in rapeseed oil and was reported to cause cardiac lesions, lipidosis, growth retardation, and increased cholesterol levels in rats. The first low erucic acid rapeseed (LEAR) was marketed in 1969, but because glucosinolates, still present in LEAR, were linked to liver hemorrhage and thyroid enlargement in livestock, rapeseed meal was not suitable as livestock feed. In 1974, the first "double low" (low erucic acid, low glucosinolate) rapeseed cultivar was available to growers and named canola (anonymous, 1981). In 1994, canola surpassed wheat as Alberta's number one cash crop.

All *Brassica* spp. contain glucosinolates, sulfur-containing glycosides that produce a sharp taste (in humans) like that of mustard and horseradish. There are more

than 70 known glucosinolates differing only in the nature of the sidechain (Kjær, 1976). Three classes of glucosinolates occur in *Brassica* spp.: alkenyls (originating from methionine), indolyls (from tryptophan) and aralkyls (from phenylalanine) (Mithen, 1992). Glucosinolates can be hydrolyzed by the non-specific glycoprotein myrosinase which is compartmentalized subcellularly in mitochondria, dictyosoma, and smooth endoplasmic reticula (Iversen, 1970), and mobilized when tissue is damaged. Hydrolysis of alkenyl and indolyl glucosinolates produces glucose, nitriles, thiocyanates, isothiocyanates, and alcohols (Mithen, 1992).

Glucosinolates occur in all tissues of *Brassica* spp. (Mithen, 1992). Roots, stems, leaves, inflorescences, and fruits display similar qualitative patterns within a species, but absolute content varies (Kjaer, 1976). *Brassica rapa* and *B. oleracea* differ in glucosinolate content (Mithen *et al.*, 1987) and wild populations of *Brassica* spp. have higher total glucosinolate levels than cultivated non-double-low *Brassica* spp. Canola (*i.e.* double low cultivars such as *B. napus* cv. Westar) possesses ~ 10 - 20 μmoles g^{-1} glucosinolates in seeds. Glucosinolate content of seeds of *B. napus* and *B. rapa* is determined by maternal genotype (Kondra and Stefansson, 1970) and is not correlated with that of leaves except in young seedlings (Milford *et al.*, 1989). Sinigrin, also known as allyl glucosinolate, is an alkenyl glucosinolate that occurs in high concentrations in *B. juncea* and *B. oleracea* seeds and leaves, but is not found in *B. napus* cv. Midas, a low erucic acid, high glucosinolate cultivar that contains other alkenyl (progoitrin and gluconapin), indolyl (glucobracissin) and aralkyl (gluconasturtiin) glucosinolates (Sang *et al.*, 1984). Further, reduction of glucosinolates in canola is mainly due to alkenyl

glucosinolate reduction, and only small differences in concentrations of indolyl glucosinolates occur between high and low glucosinolate cultivars (Mithen, 1992).

1.2 *Plutella xylostella* biology and state of the literature

Plutella xylostella (L.) (Lepidoptera: Plutellidae), the diamondback moth, is a cosmopolitan pest of *Brassica* spp. and attains pest status due to several characters of its biology. First, *P. xylostella* is found everywhere its host plants exist (*i.e.* Europe, Asia, Australia, Africa, and North America), and there is some evidence that it may be able to overwinter in northern temperate climates (Dosdall, 1994; Talekar and Shelton, 1993). As well, it can escape overpopulation and decreases in plant quality by 'migrating' hundreds of kilometers (Smith and Sears, 1982). Therefore, any management strategy must take into consideration both resident and immigrant populations. Second, unlike other pests of canola, diamondback moth is multivoltine (Harcourt and Cass, 1966) with as many as six generations per growing season in Canadian prairie provinces, and each female can produce an average of 250 eggs (Sarnthoy *et al.*, 1989). Relatively low population densities early in a growing season can increase exponentially to become problematic during pod development (in oilseed crops) and fruit ripening (in vegetable plantations). Third, *P. xylostella* larvae are not constrained by tissue specificity and feed on all aerial portions of their hosts. Plants that expend energy to repair vegetative structures suffer diminished reproductive success (R.J. Lamb, personal communication), and flowers destroyed by herbivory cannot produce seedpods. Seeds are susceptible to herbivory, and canola seedpods that are stripped of their photosynthetic surfaces exhibit poor seed development and shattering (R.J. Lamb, personal communication). Lastly, *P.*

xylostella develops insecticide resistance quickly. For example, *P. xylostella* exhibited complete resistance to pyrethroids and insect growth regulators in approx. 20 generations. *Plutella xylostella* was the first insect reported to exhibit resistance to *Bacillus thuringiensis* (*B.t.*) in field conditions (Talekar and Shelton, 1993), and Tabashnik *et al.* (1991) reported a 430- to 820-fold increase in resistance to *B.t.* within nine diamondback moth generations.

About 70% of *P. xylostella* literature deals with insecticide control and resistance, and this body of literature is rapidly expanding with the exploration of microbial resistance and use of transgenic cultivars. Less than 20% focuses on diamondback moth - crucifer interactions and of that 20%, approximately half investigate oviposition and nearly all are concerned with a single plant characteristic that is lacking in canola: allyl glucosinolate (*i.e.* sinigrin) or its breakdown product, allyl isothiocyanate.

Marsh (1917) concluded that *P. xylostella* larvae feed exclusively on plants with "mustard-like characteristics" (now known to be sulfur-containing glucosinolates) and oviposition increases in the presence of these, in particular, allyl glucosinolate (Reed *et al.*, 1989; Gupta and Thorsteinson, 1960). Palaniswamy *et al.* (1986) demonstrated that diamondback moths are attracted to host plant volatiles, and suggested that allyl isothiocyanate may be used by diamondback moths to locate hosts. Also, Hillyer and Thorsteinson (1969) reported that females exposed to allyl isothiocyanate deposit eggs sooner, and produce a greater number of mature oocytes, than females not exposed to the volatile. Pivnick *et al.* (1990) found that adult females emerging in the absence of host plants have delayed sexual maturation. Such a reproductive diapause predisposes this organism for migration because energy is not expended in maturation of eggs, but rather

invested in dispersal (at least initially), and because flight should require less effort in females not weighted with mature oocytes.

The effects of epicuticular waxes on *P. xylostella* oviposition have received relatively little attention. Eigenbrode *et al.* (1991) showed that larvae had higher survivorship on *B. oleracea* with normal wax blooms (waxy) than those with reduced wax (glossy). The addition of wax and n-alkanes to sinigrin-treated substrates increased oviposition activity by *P. xylostella* (Spencer, 1996). However, the means by which waxes influence the biology of this insect is not understood.

1.3 Rationale

"The potential complexity of the chemical environment in which phytophagous insects live and reproduce is staggering, as is the challenge that this presents to an insect's sensory nervous system. [An] understanding of sensory physiology and neurobiology is essential to understanding host-choice behavior, given that the observed behaviors are manifestations of underlying physiological processes."

--- William T. Wcislo, Cornell University.

1.3.1 Reproductive systems

Reproductive anatomy of *P. xylostella* has not been described in detail elsewhere and descriptions of closely related species are also sparse, making comparative studies difficult. The internal morphological discoveries of chapter two were originally intended to investigate reproductive readiness of males and females at eclosion as a preamble to mating and oviposition behaviors. However, the discovery of simple yet interdependent

genitalia led to questions of whether distant populations would retain this interdependence such that widely-separated populations would be reproductively compatible.

Because *P. xylostella* has a worldwide distribution and is a pest on economically important crops, investigators of this insect are presented with both economic promise for basic and applied research, and a cautionary flag when interpreting data. *Plutella xylostella* survives in a number of environments: at a macroscale, in cooler humid climates of Great Britain, dry temperate prairies of North America, or warm humid gardens of Australia and, at a smaller scale, on wild crucifers of the Mediterranean, market cabbage of South East Asia or canola varieties of the Canadian prairies. The extent to which environmental constraints affect individuals of a population, and how those pressures manifest themselves within an individual, may contribute to disparities (structural, morphological, behavioral, *etc.*) among *P. xylostella* populations.

Chapter two describes morphological characters of the reproductive systems of both male and female moths. At the level of the individual, this study provides an understanding of reproductive biology that includes anatomical and behavioral components; at the population level, consideration is given to possibilities of population isolation and the influences of larval diet on genitalia morphometry.

1.3.2 Behavior

Insect behavior studies usually involve 'endpoint' behaviors *(e.g.* amount of leaf consumed, number of eggs laid), likely because such studies are generally less time consuming and often not technically difficult, and because endpoints generate numeric

data that are conducive to statistical analysis. However, endpoints entail quantifiable behaviors generally involving multiple inputs and are the outcome of a sequence of behaviors. For example, ovipositing *Delia* spp. (Diptera: Anthomyiidae) land on a plant, initiate several vertical runs on the stalk of the plant that can be interrupted by circular runs around the base of the stalk, contact the soil with the labellum and eventually extend the ovipositor into the soil and release an egg (*Delia radicum*, Städler and Schöni, 1990; *Delia antiqua*, Harris and Miller, 1988). Not only are multiple inputs necessary, but stimuli can be synergistic. In the case of *Delia antiqua*, Harris and Miller (1982) reported that treatments providing only a visual (shape or color) or chemical (chopped onion) stimulus, received 3% of the total number of eggs deposited on treatments that combined all three stimuli.

While endpoint assays are useful to determine plant adequacy, they do not offer insight into mechanisms used for host identification. Unfortunately, most investigations of plant-insect interactions deal with only single characteristics of plants (labelling these 'stimulants' or 'deterrents' according to effects they have on the endpoint behavior in question), and therefore address only a minute part of the whole. While single character studies are useful preliminaries to subsequent research, the ideology is an overly reductionistic approach to a complex question: Why does this insect species choose this plant species?

The question of host plant selection has at least three parts. 1) What characteristics does the plant possess? Morphology, epicuticular components, phytochemicals including secondary compounds, and nutritional quality can influence acceptance. 2) What characteristics does the insect possess? To exploit its host, an insect

must possess abilities that enable it to find and recognize potential hosts. These include: behaviors that position the insect (or part of an insect) in a location that facilitates perception by sensory cells, sensory cells that are reactive to specific traits of the host (*e.g.* phytochemicals), and a central nervous system (CNS) that integrates internal and external cues to produce coordinated motor outputs. 3) How do this insect and this plant overlap in time and space, with each other and with other species? Phenology and location of the insect, of course, must coincide with that of its host, but there are other considerations such as community structure, host preferences and plant tissue specificity. Chapter three describes the behaviors associated with egg deposition and suggests mechanisms of oviposition site determination. Two such mechanisms are later explored in chapters five (olfaction) and six (gustation) which include descriptions of sensory elements and identifications of some plant characteristics involved.

1.3.3 Plant-Insect Interactions

Although a causal relationship is often assumed, acceptance of plant cues and suitability of plants are not necessarily correlated. For example *Euphydryas editha* (Lepidoptera: Nymphalidae) increases host range (*i.e.* the number of species accepted as a host) if preferred hosts are not available and will oviposit on substandard, perhaps unsuitable, plants (Singer, 1982). It is more precise to consider host selection to result from either of the current antithetical hypotheses: (1) that insects choose host plants on the basis of secondary chemical profiles (presence of stimulants, absence of deterrents) or, (2) that insects choose plants based on primary metabolites. However, each of these has inherent problems: secondary compounds are perhaps too numerous for consideration

by oligo- and polyphagous insects, and primary metabolites are too ubiquitous, providing no specificity for (especially) monophagous insects. Fraenkel (1959) suggested that secondary compounds are cues to relative nutritional acceptability, and this is the most likely in the case of *P. xylostella,* since *Brassica* spp. produce sulfur-containing glucosinolates, plant vigor is greater when sulfur is not limiting, and ovipositing *P. xylostella* do not prefer, nor is larval survival high on, sulfur-deficient plants.

Glucosinolates are classified as secondary metabolites. Secondary metabolites of plants initially were thought to be waste products because they were not known to have a role in primary metabolism but are now generally regarded as waste products, storage products, and/or defense agents. That low glucosinolate varieties exist suggests either glucosinolates are not waste products, or alternative pathways exist to reduce wastes in the form of glucosinolates. While there is no question they can function as defenses against herbivores, glucosinolates most likely exist foremost as storage products because:

- they contain a sulfonated glucose molecule, and both glucose and sulfur function in primary metabolism;
- crucifers possess a means to liberate glucose, the enzyme myrosinase, which is compartmentalized and made available when tissues are disrupted; and,
- indolyl glucosinolates are precursors to auxins that are required for plant growth (Mithen, 1992).

Glucosinolates, as storage products, might be adequate indicators of plant quality if they are consistently available and if they can be detected by cruciferous herbivores.

Verschaffelt (1910) first suggested glucosinolates to be feeding stimulants for the crucifer specialists, *Pieris rapae* and *P. brassicae* (Lepidoptera: Pieridae). Several

investigators have since noted glucosinolates stimulate other cruciferous insects such as *D. radicum* (Städler, 1978), *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae) (Hicks, 1974), and *P. xylostella* (Gupta and Thorsteinson, 1960). Although this family of chemicals, common to all members of Cruciferae, offers the most parsimonious exposition, it cannot be the only basis for host selection. Insect species that are pests of high glucosinolate crucifers can also be pests of low glucosinolate cultivars of *B. napus* and *B. rapa*. Of particular interest in this regard are those insects, such as *P. xylostella* and *Ceutorhynchus assimilis* (Coleoptera: Curculionidae), that feed on seeds of both high and low glucosinolate species because glucosinolate content is rated by quantity in seed, and in these cases glucosinolates can be neither a feeding stimulant nor a feeding deterrent.

That glucosinolates deter many non-crucifer specialists does not negate that they also may attract crucifer specialists. However, it is possible that crucifer specialists, in coevolutionary fashion, were attracted to some other crucifer constituent and developed a propensity for glucosinolates secondarily so that glucosinolates may not be the only compounds attracting cruciferous herbivores. Baur *et al.* (1996) isolated a non-glucosinolate fraction from cabbage, 'CIF' (cabbage identification factor), that is stimulatory to gustatory receptors of *D. radicum* and also stimulates oviposition by this insect. Spencer (1996) found that *P. xylostella* deposits more eggs on substrates treated with a glucosinolate (sinigrin) and waxes than on substrates treated with only the glucosinolate, and *P. xylostella* will deposit eggs on Parafilm® that has not been treated with any other substance (Chapter 4).

Insect species that appear to be stimulated to oviposit by glucosinolates do not penetrate the epicuticular layers of their host plants when performing oviposition behaviors (*e.g.* foretarsal drumming of *P. brassicae*; Ma and Schoonhoven, 1973). It is not yet understood if or how glucosinolates, which are polar compounds, travel through non-polar epicuticular layers. There have been suggestions that glucosinolates traverse apolar phylloplane layers via specific channels in the wax layers of the cuticle, or by means of carrier molecules, but neither mechanism has been characterized. Although the plant cuticle is the physical interface between insect and plant, it is an important component that has been, for the most part, overlooked in crucifer-diamondback moth investigations. Chapter four characterizes some phylloplane attributes and their effects on *P. xylostella* oviposition.

1.3.4 Sensory Physiology

Städler (1982) asserts that the central nervous system (CNS) is difficult to work with because of its complexity. The CNS is often referred to as a black box in which intrinsic physiology and extrinsic sensory information are registered, and specific behaviors or modifications of those behaviors supervene. Processes between and among numerous neurons and interneurons of the brain, suboesophageal, thoracic, and abdominal ganglia of insects are not well understood, and current methods allow us to look at very few of these interactions simultaneously. Advances in technology make possible more exact and more invasive research of peripheral and central nervous systems, but not yet concurrent analysis of several components. Because chemically-mediated behaviors at the CNS level are extremely difficult to investigate, sensory

systems have been primary research foci as prerequisites for understanding sensory coding in the CNS.

Chemoreception studies include detailing number, type, sensitivity, and specificity of chemosensors. Electron microscopy has provided increasingly better resolution of structures, electrophysiological techniques have been employed since the 1950's, and gas chromatography, relatively neoteric, has allowed separation and identification of active components of complex volatiles.

Insect olfactory sensilla, found mainly on the antennae and palps (Schneider, 1987) have been classified based on their size and shape (Schneider and Steinbrecht, 1968): sensilla trichodea (hair-like), sensilla basiconica (cone-like), sensilla coeloconica (peg-pits), and sensilla placodea (pore-plates). These sensilla bear 3 000 - 15 000 pores, which are 10 - 50 nm in diameter and attach to a labyrinth of tubules known as pore kettles (Metcalf and Metcalf, 1992).

Gustatory hairs can be found on mouthparts, antennae, tarsi, ovipositor, and other body parts (Schneider, 1987). Their morphology is either trichodeal (hair-like) or styloconical (peg-like), and they possess one terminal pore that is much wider than those of olfactory hairs (Schneider and Steinbrecht, 1968). Sugar- and salt-sensitive cells within gustatory sensilla are common, but few cells specific to any phytochemical stimulus have been found (Schneider, 1987). Although it has been suggested that secondary compounds are used for recognition of a host, there is little evidence for this; however, because taste is generally considered the final step in feeding and oviposition, it may be that taste is the modality ultimately responsible for acceptance or rejection of any potential host.

Insect chemosensilla (*i.e.* both olfactory and gustatory) are innervated by bipolar primary sensory neurons (up to 60 in olfactory, 2-8 in gustatory; Altner and Prillinger, 1980) that act as transducers, converting chemical stimuli to electrical signals ultimately destined for the CNS. The basis of signal transduction, similar for both gustatory and olfactory cells, takes place within the membrane receptor.

The resting membrane potential of insect neurons is slightly negative (tens of mV) with respect to outside the cell (Aidley, 1971). Adsorption of a stimulus molecule to a membrane-bound receptor of the dendrite creates a local change in membrane permeability allowing the transport of ions across the membrane. The electronic spread of the receptor potential (either depolarizing or hyperpolarizing) is subject to attenuation (*i.e.* length constant related to resistances of membrane, and inner and outer cellular fluids) as the receptor potential travels down the dendrite toward an electrogenic zone (*i.e.* the spike initiation zone). A depolarization (*i.e.* Na^+ in and Cl^- out), if sufficient in strength at its arrival at the electrogenic zone, will result in the initiation of action potentials.

The membrane receptor concept has several assumptions (Metcalf and Metcalf, 1992):

- ligand-receptor binding is reversible;
- ligand-receptor binding is saturable;
- receptors of a single type are identical and independent; and,
- binding does not alter the ligand, but modifies receptor activity.

Receptor specificity can be influenced by a number of variables including binding affinities and signal modulation via second messenger systems (see Dodd and Persaud,

1981). Chemosensory cells are often termed "specialist" or "generalist" cells based on number of receptors per cell of a single receptor type, and the number of stimuli to which a receptor type responds (*i.e.* receptor specificity) (van der Pers, 1980). Specialist cells respond to few chemicals but are highly sensitive to them; generalist cells respond to a wider array of compounds but are not specifically tuned to any particular one compound (Schneider and Steinbrecht, 1968).

Sensory cell responses are comprised of serial action potentials and can be phasic/tonic (bursts of rapid firing action potentials at stimulus onset lasting a few msec and slowing or altogether ceasing) or tonic (regular firing of action potentials separated by msec and usually lasting much longer than phasic responses). These coded messages are sent to various representatives of the CNS: olfactory information from the antennae to the deutocerebrum; gustatory information from the mouthparts to the suboesophageal ganglion, from the antennae to the dorsal deutocerebrum, and from the tarsi to respective thoracic ganglia. Deciphering these coded messages of sensory cells should be eased by the relative simplicity of insect nervous systems in that insect sensory neurons are primary neurons (Dethier, 1978). Three hypotheses of encoding have been put forth (Städler, 1984):

- labelled lines in which a single neuron carries a message to a unique identifiable locus in the CNS;
- temporal patterns in which the duration of the phasic response, or the spike intervals during the tonic phase, are specific to a single stimulus; and,
- across-fibre patterns in which a spatial pattern of activity (*i.e.* from more than one cell) provides an identifiable model unique to specific compounds.

Sorting out pattern precedence is difficult, and host plant choice is likely a multi-component system in which patterns from many sensilla are involved.

The final two chapters isolate two sensory modalities that ovipositing moths use in host determination. Chapter five utilizes gas chromatography coupled with electroantennograms (GC-EAG) of whole animals subjected to headspace odor of host and non-host plants. Chapter six investigates host plant selection further using standard tip-recording techniques of contact chemoreceptors.

1.3.5 General

With the exception of physiological mechanisms associated with insecticide resistance, little is known of the basic biology of this important crop pest, *P. xylostella*. This dissertation, in its entirety, is intended to bring to the forefront some understanding of basic biology of *P. xylostella* and its interactions with host plants, for philosophical and practical application. Given our current understanding of agricultural systems, it should be apparent that application of pesticides, in the form of spray or transgenic crop, is not effective and resistance is inevitable. Because the immature stage of *P. xylostella* has poor mobility and first instars are obligatory miners to prevent desiccation, it is an ideal candidate for prophylactic control: prevention of larval entry to plant tissues, or prevention of egg deposition by adult females. Complete exclusion is impractical but identifying what is attractive to ovipositing moths will be useful in developing traps/trapcrops and in engineering cultivars that are less attractive than their wild counterparts.

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Chapter 2:

Reproductive morphology, copulation, and inter-population variation of *Plutella xylostella* genitalia.

2.1 Introduction

Although *P. xylostella* (L.) is an economically important worldwide pest of cruciferous crops, knowledge of its reproductive anatomy is incomplete. In fact, there are few morphological studies of reproductive systems of any members of Plutellidae or the closely related Yponomeutidae, severely limiting comparisons with other Lepidoptera. Male and female external genitalia of *P. xylostella* have been described by Moriuti (1977), and superficial and inadequate descriptions of internal structures were made by Yang and Chow (1978).

Plutella xylostella is thought to have a worldwide distribution though little attention has been given to potential morphological or behavioral differences among populations. Intraspecific mating incompatibility has been reported in several widely distributed species including *Choristoneura fumiferana* (Lepidoptera: Tortricidae) (Lorimer and Bauer, 1983) and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Pashley and Martin, 1987). Comprehension of reproductive isolation within widely occurring, economically important species is important for consideration of introduction/migration and adaptation, especially with respect to genetic variability and resistance.

The purpose of this study is two-fold: to better describe the morphology of the internal reproductive structures of male and female *P. xylostella*; and, in characterizing

the concomitant precision of male and female genitalic structures, to describe variations in genitalic morphology that may function as mechanisms of reproductive isolation among distantly separated populations.

2.2 Materials and Methods

2.2.1 Insects

Adult moths were collected at Vegreville, Alberta, to establish laboratory colonies. *Plutella xylostella* were reared in the laboratory on *B. napus* cv. Westar (27°C, 70% R.H., 16L:8D), and supplemented with a mixture of honey and water. Colonies were periodically augmented with wild-caught specimens from central Alberta. Pupae were removed from rearing cages and placed singly in glass vials. Sex was determined upon eclosion. (See 2.2.4 for moths used in population comparisons.)

2.2.2 Internal morphology

Excised abdomens of teneral moths were fixed in Bouin's under vacuum and scales were subsequently removed with a camel-hair brush. Fixed specimens were dehydrated in an ethanol series to 100 percent and embedded in Paraplast Plus® (56°C with dimethyl sulfoxide; Oxford Labware, USA). Transverse and saggital sections (5 µm) were mounted on glass slides and stained with Mallory's triple stain (Barbosa, 1974). (See Appendices I-1,2 and II-1,2 for protocols.)

Serial sections of entire abdomens were photographed with slide film. Photomicrographs were projected onto paper and traced. Serial tracings were aligned and assembled into one drawing to create a three-dimensional reconstruction in which the left lateral face of cuticle is cut away at the dorsal and ventral midlines to expose internal

structures of the reproductive system *in situ*. For the sake of clarity, only the left member of paired structures was drawn in the male reconstruction. Complementary line drawings were prepared from dissections of unfixed teneral adults to show the reproductive system in its entirety.

2.2.3 Copulatory behaviors

Adult moths were observed (directly and videotaped for later inspection) just prior to the scotophase, when peak mating activity occurred. Behaviors were briefly described.

2.2.4 Genitalic configurations

Male and female specimens from Wädenswil (Switzerland), Victoria (Australia), and Hangzhou (China) were collected from pan traps baited with allyl isothiocyanate. Specimens from Vegreville, Alberta (Canada), Loxahatchee, Florida (USA), and Geneva, New York (USA) were taken from laboratory colonies that had been established from wild-caught animals the previous year. Specimens from Wageningen (Netherlands) were taken from a twenty year old laboratory colony. All specimens were preserved in 70% ethanol.

Abdomens of virgin and mated males and females were critical point dried (Appendix III-1), sputter-coated with gold (50 Å), and viewed with a JSM-6301FXV field emission scanning electron microscope. Aedeagi were dissected and viewed with light and scanning electron microscopy. Bursal ducts (including external cuticular processes) were removed from females, cleared in cold 10% KOH, and viewed using light microscopy. Measurements were made of digitized images of aedeagi and bursal ducts.

2.3 Results

2.3.1 Internal morphology

2.3.1.1 Female

Copulatory structures:

A posterior cuticular process ca. 130 μm in length arises from abdominal sternite six. This process, normally entirely obscured by scales, houses the ostium bursa, an ovoid opening measuring ca. 4.0 μm in width, and part of the bursal duct (Plates 2-I, 2-VI.3, and 2-VI.5). The bursal duct (bd) is lined with sclerotized cuticle for approximately 60 percent of its length (Plate 2-II.1).

The bursa copulatrix (bc), located ventral to the alimentary canal in segments five and six, is membranous and large. It is collapsed prior to mating and round after mating (up to 450 μm diam) (Plates 2-I, 2-II.1, and 2-II.2). Its epithelial cells are irregularly shaped with round nuclei (2.7 μm diam) (Plate 2-II.2). Dissections of teneral and mated females revealed a transparent bursa copulatrix in which signa were absent. The cuticle of the bursa copulatrix is of uneven thickness and bears numerous cuticular ridges on the inner surface. In inseminated females, there was no evidence of a spermatophore and in dissections of unfixed specimens, freely-swimming sperm were visible within the bursa copulatrix. An infundibulum leads to a seminal duct that leads through the common oviduct to the opening of the spermatheca (Plate 2-II.1).

The tubular spermatheca (sp) lacks a lagenar arm but, because of its length (ca. 1.5 mm), loops first posteriorly and then anteriorly (Plate 2-I). In cross section, small columnar epithelial cells are arranged about a keyhole-shaped lumen with two distinct canals that open into each other mesially (Plate 2-II.3). The smaller is lined with heavily

sclerotized cuticle and has an inner diam of < 3.0 μm. A thick layer of circular muscle (mu) surrounds the spermatheca; longitudinal muscle is also present external to but less obvious than the circular muscle.

An oblong spermathecal gland (spg) is present at the distal end of the spermatheca (Plates 2-I and 2-II.1). Large, irregularly shaped cells with homogeneous cytoplasm, large nuclei (6.3 μm diam) and secretory vesicles (<2.0 μm), surround an extremely narrow duct (4.5 μm diam) that is lined with cuticle. Cells appear slightly separated from each other.

Ovarioles and primary exit system:

There are four polytrophic ovarioles (or) in each ovary and seven nurse cells per oocyte. Ovarioles lack a common surrounding sheath to form a true ovary (Plates 2-I and 2-II.1). Adults emerge with previtellogenic and vitellogenic oocytes, and therefore ovarioles occupy a large part of the abdomen. Ovarioles are 3.3-3.9 mm from pedicel to terminal filament, fold back on themselves in the second abdominal segment, and are suspended from the dorsal abdominal wall by connective tissue. Terminal filaments (tf) of each ovary are twisted together to form a complex of tissue not easily separated in dissections.

Abundant folds in the epithelial lining of the pedicels, and lateral and common oviducts, increase posteriorly. Their epithelial cells are cuboidal (11.7 x 9.0 μm) and have round nuclei (5.4 μm diam) basally. Genital and rectal tracts merge to form a common exit (*i.e.* an 'incomplete cloaca'; see Dugdale, 1974) immediately prior to the external opening.

A dorsal ovipositor is formed by a pair of lobes (Plates 2-I, 2-VI.3, and 2-VI.5), and the external surface of each lobe possesses numerous uniporous and aporous sensilla (Chapter 6). The entire structure is usually partially withdrawn into segment seven and obscured by scales except during egg deposition (Chapter 3), at which time the ovipositor is protracted by contraction of protractor muscles inserted into four posterior apophyses (two ventral and two dorsal; not shown).

Colleterial gland:

A bilobed colleterial gland (cg) occurs at abdominal segment six and tapers posteriorly to empty into the base of the common oviduct (Plates 2-I and 2-II.1). The lumen of each lobe is ca. 44 μm at its widest, and is filled with a material that stains dark blue with Mallory's triple. Its columnar epithelial cells (19.0 x 5.0 μm) have grainy cytoplasm and large (4.5 μm diam) round nuclei situated basally (Plate 2-II.4). Secretory vesicles (<1.8 μm diam) are present apically and stain like the material in the lumen. The entire structure is surrounded by a thin layer of longitudinal muscle.

2.3.1.2 Male

Testes and vasa deferentia:

Testes (ts) are fused into a sphere (ca. 337 μm diam) and are situated dorsally in abdominal segments three and four (Plates 2-III and 2-IV.1). Eight sperm tubes, or follicles, are incompletely separated by thin follicular membranes and the entire structure is surrounded by basal membrane encased within a crystalline-like layer of unknown composition. Cysts containing primary spermatocytes and developing spermatids or mature sperm are easily identifiable within follicles.

Mature sperm bundles enter the vasa deferentia (vd) through one of two postero-dorsal ostia (9.5 μm diam) (Plates 2-III and 2-IV.1). The proximal vasa deferentia (*i.e.* 'seminal vesicles'; see Callahan, 1958) are bulbous and are delimited by large (9.0-16.0 x 6.5 μm) columnar cells with spherical nuclei (3.5 μm diam) at the apical end. The lumina of the vasa deferentia measure 80 μm at their widest section, and each duct tapers to a delicate tubule (30 μm outer diam). This distal part of the vasa deferentia has cuboidal epithelial cells (7 x 7 μm) with elliptical nuclei in cross section. Both proximal and distal vasa deferentia are encased in a thin layer of muscle, and the lumina of both contain mature spermatozoa embedded in flocculent material.

Accessory glands:

Accessory glands (ac) are tubular, paired, and fused distally; the right and left glands end in a constriction followed by a spherical dilation (Plates 2- III and 2-IV.1), and these dilations (di) are joined by a short (ca. 30 μm), narrow (25 μm outer diam) tubule with no apparent septum. Lumina diameters of the right and left accessory glands, and those of the spherical dilations, measure 35 μm and 60 μm, respectively. The entire structure is lined with cuboidal epithelial cells (8.0 x 11.0 μm) that have flat, elliptical nuclei in cross section (2.7 x 6.3 μm) and dense cytoplasm (Plate 2-IV.2) containing small refractive granules near the apical borders. Because of their length (ca. 1.9 mm), left and right glands run anteriorly along the ventral side of the testes and fold back upon themselves so that spherical dilations *in situ* are located at abdominal segments four and five (Plate 2-III).

Ejaculatory ducts and aedeagus:

Accessory seminal vesicles (asv), a pair of large bulbous structures (lumen is 120 μm at the widest point) located side by side immediately posterior to the testes, are convergence zones for accessory glands and vasa deferentia, the former entering anteriorly and the latter ventro-posteriorly (Plates 2-III and 2-IV.1). Individual spermatozoa and accessory gland material are apparent in the lumina of these vesicles. Epithelial cells are columnar (13.0-16.5 x 5.5 μm) with large round nuclei (6.3-7.2 μm diam). Thin layers of circular (inner) and longitudinal (outer) muscle are present.

The ejaculatory duct duplex (edd) is formed by a short tubule (0.4 mm), exiting the dorsal side of each accessory seminal vesicle, and continuing posteriorly along the dorsum from the fourth to the fifth abdominal segment. The lumen (2.4 μm diam) of each arm of the duplex contains material similar to but less dense than that of the accessory seminal vesicles. The columnar cells (18 x 5.3 μm) of the ejaculatory duct duplex have grainy cytoplasm and round (3.8 μm diam), basally-situated nuclei.

These two ducts converge posterioly at mid-fifth abdominal segment to form the ejaculatory duct simplex (eds) (Plates 2-III and 2-IV.1). Thin layers of circular (inner) and longitudinal (outer) muscle surround both ejaculatory duct duplex and simplex. Columnar cells of the simplex (9.0-10.8 x 3.6 μm) have basally-situated round nuclei (3.2 μm diam) and grainy cytoplasm containing secretory vesicles and refractory granules. The diameter of the lumen is ca. 19 μm throughout its length. The simplex is relatively long (1.2 mm) and loops ventral to the saccus in the fifth abdominal segment (Plate 2-III). The anterior portion (ca. 70 percent of total length) contains flocculent material and sometimes individual spermatozoa, and nuclei of epithelial cells are chaotically situated

through (*i.e.* they are neither strictly apical nor strictly basal); the remainder of the duct, or posterior portion, contains homogeneous material in which spermatozoa are not apparent in teneral adults, and epithelial cells are distinctly columnar with basal nuclei (Plate 2-IV.3).

At the junction of the ejaculatory duct simplex - cuticular simplex (cs), the outer diameter of the duct is 31.6 μm and its narrow lumen (11.5 μm) is lined with cuticle. Epithelial cells appear degenerate and circular (inner) and longitudinal (outer) muscle is apparent. This pattern continues posteriorly for ca. 0.30 mm, at which point circular muscle greatly thickens (Plates 2-III, 2-IV.1, and 2-IV.4). The outer dimensions widen and flatten laterally (67 x 115 μm) but the luminal diameter remains constant. The structure makes a single fold, with the lumen following the outer edge of the fold (ca. 10 μm from the outer edge of the fold). Circular muscle lessens (*i.e.* outer dimension narrows) toward the base of the aedeagus (Plates 2-III, 2-IV.1, and 2-IV.4).

The aedeagus (ad) is a simple chitinous needle-like structure; the ostium angle is approximately 45° (to the transverse plane) so that the aperture measures 20 μm x 3.5 μm. Two lateral basal processes for muscle attachment are apparent. Cornuti are absent.

2.3.2 Copulatory behaviors

Plutella xylostella, like most Lepidoptera, display end-to-end mating. A sexually active male, when less than 1 cm from a female, fans its wings and turns its body so that the tip of its abdomen approaches the potential mate. At the same time, the harpes abduct laterally to expose two tufts of hair pencils (one on either side of the abdomen). If the female is receptive, she remains and the male backs into her, with the harpes grasping lateral sclerites of the female during copulation. Non-receptive females move away from

wing fanning males. Once paired, wing fanning ceases and the male re-folds its wings to rest over the back of the female (Plate 2-V). Pairs remain *in copula* for longer than two hours.

2.3.3 Genitalic configurations

The aedeagus fits into the bursal duct during copulation and this fit is highly specific in angle, shape, and size (Plate 2-VI.1): the diameter of an individual spermatozoa is 2.9 μm; the inner diameter of the aedeagus is ca. 3.5 μm; the outer aedeagal diameter (measured at 2/3 length) is ca. 4.0 μm; and, the inner diameter of the bursal duct is ca. 4.0 μm.

Scanning electron micrographs of the ostium bursa of virgin and mated females are distinctly different with the latter apparently obstructed (Plate 2-VII). No differences were noted between virgin and mated males.

Although overall body size did not differ among populations (Fig. 2-1), aedeagi were quite variable in length: Canadian specimens possessed aedeagi that were significantly shorter and those of Australian specimens significantly longer (ANOVA, $\alpha = 0.05$, $P < 0.0001$, Duncan's post hoc test) (Fig 2-1; Plate 2-VIII) than other specimens (with New York having the second longest).

Lengths of the posterior cuticular process of females did not differ among populations, but the total lengths of the bursal ducts (including posterior processes) were significantly different among populations (ANOVA, $\alpha = 0.05$, $P < 0.0001$, Duncan's post hoc test) (Fig. 2-2) and similar to adeagal results. Specimens from Canada and the Netherlands had short ducts and specimens from Australia possessed long ducts but not significantly longer than those of specimens from China, New York, or Switzerland.

Also, specimens from Australia had ducts that were markedly more sclerotized than others (Plate 2-IX).

With the exception of the two extremes (Australia and Canada), measurements of aedeagal lengths and bursal ducts were not strongly corresponding across populations.

2.4 Discussion

Many lepidopteran reproductive systems, particularly those of economic pests, have been described: *Ephestia kühniella* Zeller (Pyralidae; Musgrave, 1937), *Diatraea grandiosella* Dyar (Pyralidae; Davis, 1968), *Heliothis zea* (Noctuidae; Callahan, 1958), *Pseudoletia unipuncta* (Haworth) (Noctuidae; Callahan and Chapin, 1960), *C. fumiferana* (Clemens) (Outram, 1970, 1971), and *Cydia pomonella* L. (as *Laspeyresia pomonella* (L.), Tortricidae; Ferro and Akre, 1975). Both female and male *P. xylostella* display typical lepidopteran reproductive systems with some unique characteristics.

Ovariolar morphology in this moth is typical of Lepidoptera. Teneral females possess oocytes at varying stages of development up to and including vitellogenesis, but none are chorionated upon eclosion. *Plutella xylostella* has a 1-3 day pre-oviposition period (Hillyer and Thorsteinson, 1969) and presumably egg chorionation takes place during this time. Because of this perpetual oocyte development and variability among females, no attempt was made to determine number of eggs produced per female. The excessive folds of the lateral and common oviducts likely accomodate the passage of oocytes during oviposition. The occurrence of a complete or incomplete cloaca is a

lepidopterous condition (Dugdale, 1974). Incomplete cloacas are typical of suborder Dytrisia and *P. xylostella* is no exception.

It appears that sperm movement is 'streamlined' in this system; sperm pass from the bursa copulatrix to the spermatheca via a seminal duct that is distinct even within the common oviduct. A similar phenomenon occurs in females of *Diatraea saccharalis* (F.) and is theorized to reduce sperm loss during transport (Miskimen *et al.*, 1983).

Yang and Chow (1978) described a white, opaque, balloon-like structure, the spermatophore corpus, within the bursa copulatrix shortly after a female paired with a male. However, no such structure was visible in my dissections of unfixed females 30 minutes and 120 minutes post-pairing, even though the bursal wall is transparent and freely mobile sperm were observed. George and Howard (1968) reported that male *Grapholitha molesta* (Tortricidae) produced progressively smaller and eventually no spermatophores with subsequent matings. This cannot be the explanation for a lack of spermatophores in my investigations because only new emergents were used and were allowed to pair only once. *Plutella xylostella* appears to fit the "second female-determined method" (Gerber, 1970) of spermatophore formation in which materials are passed to the female during copulation and these materials do not encapsulate the spermatozoa, but harden in the copulatory duct to form a mating plug. Scanning electron micrographs of virgin and mated females confirm the existence of such a formation in mated *P. xylostella* in western Canada. Further research is necessary to determine if spermatophore formation differs among populations.

Plutella xylostella is similar to *Hepialis humuli* L. (Lepidoptera: Hepialidae) in that bursal signa and bulla seminalis are not present (Pyatin, 1989). Also the spermatheca

of *P. xylostella* is tubular and lacks a lagenar arm as in *H. humuli* (Pyatin, 1989) and *C. fumiferana* (Outram, 1971).

The dual canal system of the spermatheca is similar to that described in *C. fumiferana* by Outram (1971) and in *D. saccharalis* by Miskimen *et al.* (1983) in which sperm pass through the larger canal of the spermatheca and migrate singularly down the smaller canal to fertilize oocytes. In many Lepidoptera, either or both of these spermathecal canals exhibit two or more spirals (*e.g. D. saccharalis,* Miskimen *et al.*, 1983; *C. pomonella,* Ferro and Akre, 1975; *Laspeyresia caryana* (Fitch), (Tortricidae), Tedders and Calcote, 1967; and *E. kühniella,* Musgrave, 1937), but no such spirals occur in *P. xylostella.*

The purpose of a spermathecal gland in Lepidoptera is unknown but it may produce an exogenous nutrient source for spermatozoa. Little seminal fluid is passed from male to female during copulation and is likely insufficient to maintain sperm for the duration of storage in the spermatheca (Smith, 1968).

The colleterial gland of *P. xylostella* is not comprised of reservoir and thread-like extensions as in *C. pomonella* (Ferro and Akre, 1975), *C. fumiferana* (Outram, 1971), *D. saccharalis* (Miskimen *et al.*, 1983) and *D. grandiosella* (Davis, 1968). Instead, a single structure appears to function both as a production site and reservoir. Products of the colleterial glands of Lepidoptera are thought to be adhesives used to glue eggs to substrate during oviposition. That this product in *P. xylostella* stains blue with Mallory's triple suggests it is a mucopolysaccharide, but the nature of this material was not investigated further.

Testicular structure in males is typically lepidopteran with incompletely separated follicles in one fused, spherical testis (*e.g. C. pomonella,* Ferro and Akre, 1975; *C. fumiferana,* Outram , 1970; *D. grandiosella,* Davis, 1968; *L. caryana,* Tedders and Calcote, 1967; and *P. unipuncta,* Callahan and Chapin, 1960).

Flocculent material of the proximal vasa deferentia may sustain sperm while they are stored and is likely secreted by columnar cells although no secretory vesicles were visible using light microscopy.

Vasa deferentia, accessory seminal vesicles, and ejaculatory ducts appear similar to those found in representative noctuids (*P. unipuncta* and *Peridroma margaritosa* (Haworth), Callahan and Chapin, 1960), tortricids (*C. fumiferana*, Outram, 1970) and pyralids (*D. grandiosella*, Davis, 1968). There are two distinct content types within the lumen of the ejaculatory duct simplex in *P. xylostella*, one appearing flocculent and the other, at the distal end, homogeneous. While Davis (1968) characterized two distinct regions of the ejaculatory simplex of *D. grandiosella* and Outram (1970) described seven regions in *C. fumiferana* based on lumen contents, it is not known if this is a species-specific character or if the number of regions is biased by technique employed.

The accessory glands are of particular interest since the distal ends of the two are joined. Such a configuration has not been reported in other Lepidoptera. In *E. kühniella, C. pomonella, C. fumiferana,* and *L. caryana*, the left and right glands are associated with each other lengthwise but remain as separate units (Riemann and Thorson, 1979; Ferro and Akre, 1975; Outram, 1970; and Tedders and Calcote, 1967; respectively). The dense uniform cytoplasm of epithelial cells is the same throughout the lengths of the accessory glands, as is the lumen content, and suggests production of a single or only a few

products, unlike glands of *E. kühniella* (Musgrave, 1937), *H. zea* (Callahan and Cascio, 1963), and *C. fumiferana* (Outram, 1970), which can be divided into distinct regions based on gross morphology and luminal contents. The products of male lepidopteran accessory glands are spermatophore precursors and/or spermatozoal nutrients, both of which are passed to the female during copulation. It is likely that the product of the male accessory glands of *P. xylostella* functions only as the latter because the bursa copulatrix contains no obvious spermatophore (*i.e.* corpus, frenum, or collum). Although lack of a septum between left and right glands is puzzling, anastomozing muscles at the distal constrictions may be sufficient to maintain unidirectional flow of accessory gland fluid.

Copulation of *P. xylostella* appears similar to other moth species with no obvious female behavior. The hair pencils displayed by the male appear to be similar in form and function to those of *Acrolepia assectella* described by Thibout (1972) and play a role in pre-copulatory behavior. Hair pencils (and other extrusible organs) of male moths are dispensers of short-distance pheromones used for courtship (Birch and Hefetz, 1987). Wing fanning generates air currents that pass over hair pencils to help disperse volatile pheromones.

Aedeagal cornuti and bursal signa are lacking in *P. xylostella* and therefore are not required for a 'locking mechanism' for copulation. Rather, simple and penurious fits between aedeagus and bursal duct (including the posterior cuticular process), along with pheromones and species-specific behaviors, adequately serve to maintain reproductive isolation within this species.

The sclerotization of the bursal duct is advantageous over a membranous duct to prevent breakage during copulation (considering the size, shape, and strength of the aedeagus). Bursal ducts in all populations were shorter than corresponding aedeagi but it is probable that the aedeagus does not completely penetrate the duct during copulation (considering outer diameter of the aedeagus at 2/3 its length and inner diameter of the ostium bursa) in which case aedeagal measurements, albeit consistent among specimens, overestimate the particular length of aedeagus involved in copulation. The heavy sclerotization of the ductus bursae in Australian specimens could be an adaptation to accomodate the extra size of the aedeagus of males of this population.

Because of these morphometric incongruences among populations, it would be interesting to attempt crosses between the Australian population with (for example) the Canadian population to see if incompatabilities exist and whether they are unidirectional. If heavy sclerotization of the bursal duct is required for accomodating larger aedeagi, crossing males from Canada with females from Australia would be feasible, but the reciprocal cross should not be possible.

Irrespective of potential reproductive isolation, differences among populations are of interest because there must also be a mechanism effecting these discrepancies. One possibility is host plant variability. Begum *et al.* (1996) discovered several morphometric characteristics (*e.g.* femur and wing lengths) that differed with larval host plants. Larvae reared on young cabbage, *B. oleracea* cv. capitata, always showed larger body tagma than those reared on two wild crucifers, indian marshcress, *Rorippa indica*, and Virginia pepperweed, *Lepidium virginicum*.

Morphometric results of this study appear to be consistent with Begum's host plant hypothesis. Field caught specimens from Australia originated in cabbage gardens, and those from Switzerland and China originated from crucifer vegetable gardens in which cabbage was included. Both laboratory-reared colonies from the USA were reared on young cabbage plants. Laboratory colonies from Canada and the Netherlands were reared on various *Brassica* spp. including *B. napus* cv. Westar. If the morphometric-host plant relationship is equivalent to a morphometric-nutrition relationship, then *B. napus* must be of suboptimal nutrition like that of *R. indica* and *L. virginicum*. Given this, it should be possible to obtain specimens from Australia that have shorter aedeagal lengths.

Perhaps sclerotization of the bursal duct is also a result of larval diet, perhaps as waste regulation rather than reproductive adaptation. This would require the sequestration of some cuticle precursor (*e.g.* glucosamine) and the occurrence of such precursors would have to vary in amount with host plant species, be ingested by larvae, not used in other biochemical pathways, and not excreted before or after pupation but shunted to cuticle synthesis. The likelihood of this is marginal, especially given the high energy requirements for and utilization of glucosamine by insects (R.H. Gooding, personal communication).

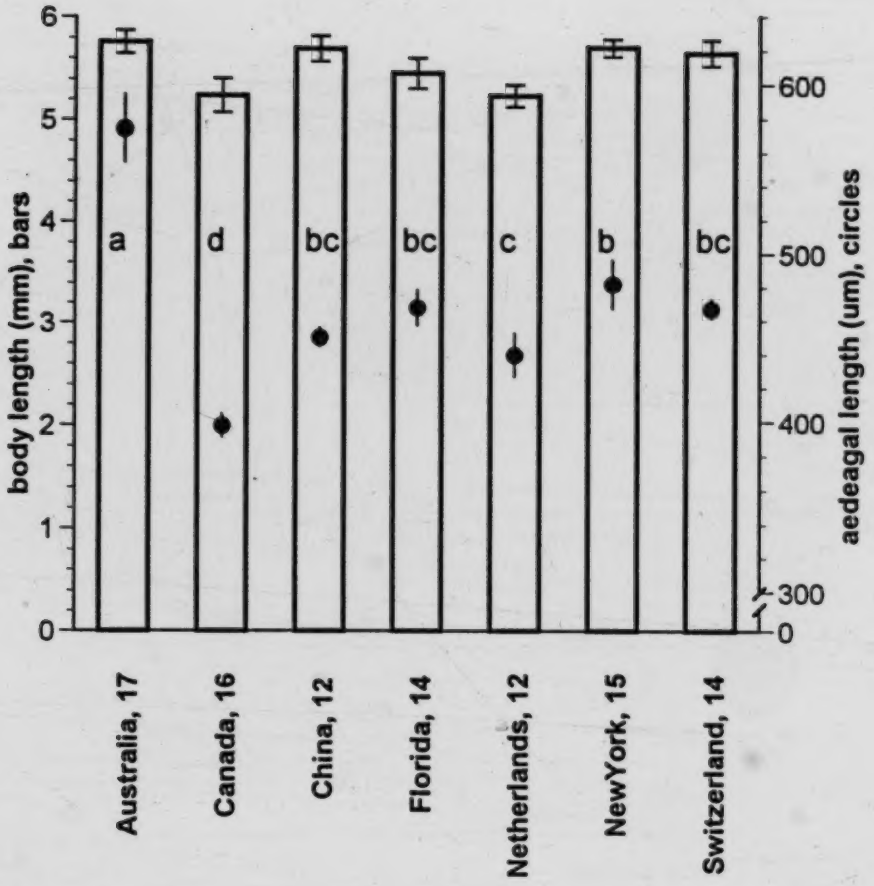

Figure 2-1. Body length, excluding wings and antennae (bars, left axis) and aedeagal length (circles, right axis) ± standard errors, of specimens from seven populations; sample sizes are given in column labels; letters represent significant differences of aedeagal lengths only (one-way ANOVA, $\alpha = 0.05$, $P < 0.0001$); body sizes are not significantly different.

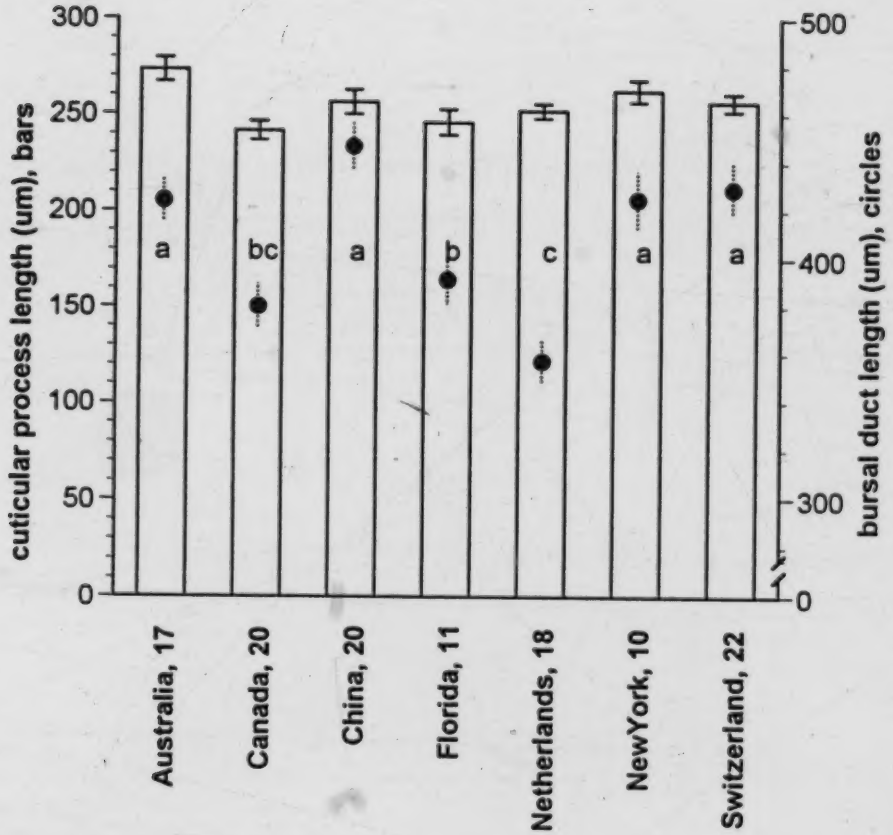

Figure 2-2. Posterior cuticular process lengths (bars, left axis) and bursal duct lengths (circles, right axis) ± standard errors, of specimens from seven populations; sample sizes are given in column labels; letters represent significant differences of bursal duct lengths only (one-way ANOVA, $\alpha = 0.05$, $P < 0.0001$); cuticular process lengths are not significantly different.

Plate 2-I. Reconstruction of internal reproductive structures of female *P. xylostella*. Left lateral cuticle is excluded between ventral and dorsal midline to expose structures *in situ*. Roman numerals denote abdominal segments; bar = 500 μm.

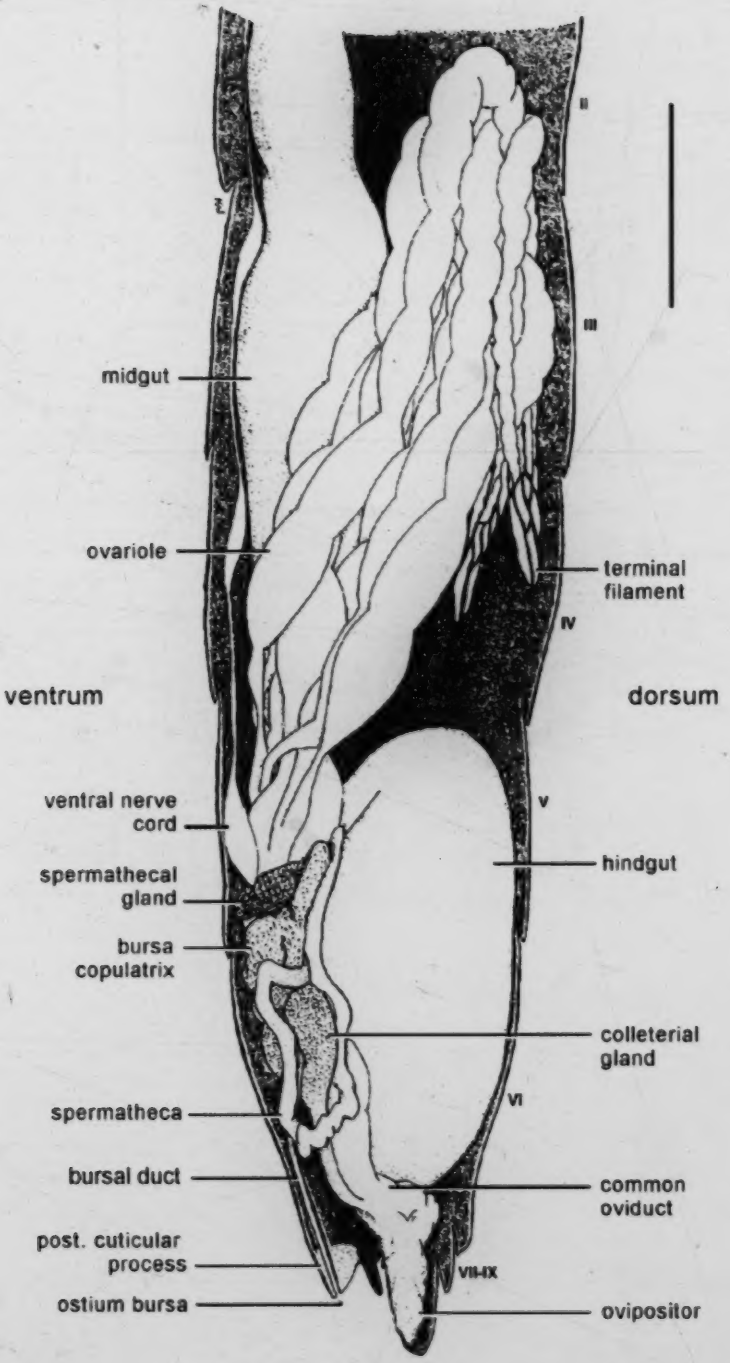
II
III
midgut
ovariole
terminal filament
IV
ventrum
dorsum
ventral nerve cord
V
hindgut
spermathecal gland
bursa copulatrix
colleterial gland
VI
spermatheca
bursal duct
common oviduct
post. cuticular process
VII-IX
ostium bursa
ovipositor

Plate 2-II. 1) Line drawing of female reproductive structures, note differential cuticle thickness of the bursal duct, bar = 400 μm; 2) Sagittal section of the bursa copulatrix of an unmated female, bar = 50 μm; 3) Transverse section through spermatheca, bar = 50 μm; 4) Sagittal section through bilobed colleterial gland, bar = 50 μm. Abbreviations: **bc** - bursa copulatrix, **bd** - bursal duct, **cg** - colleterial gland, **ct** - cuticle, **fb** - fat body, **lu** - lumen, **mu** - circular muscle, **n** - nucleus, **od** - oviduct, **or** -ovariole, **pd** - pedicel, **sd** - seminal duct, **sp** - spermatheca, **spg** - spermathecal gland, **tf** - terminal filament.

1

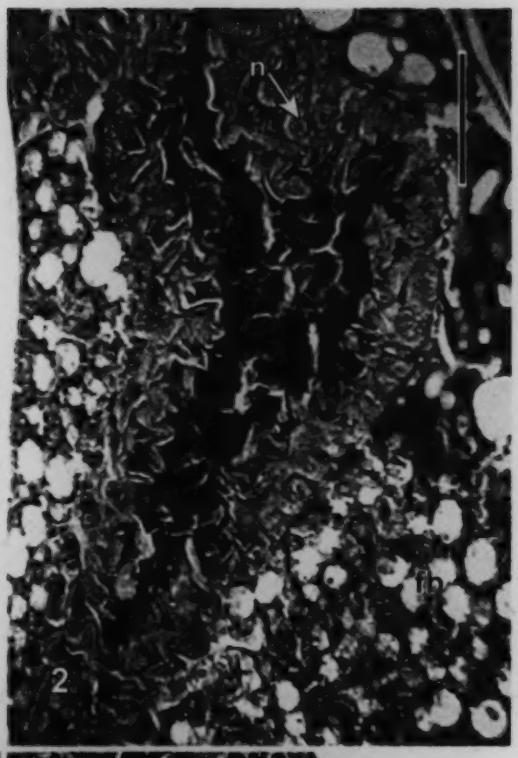

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Plate 2-III. Reconstruction of internal reproductive structures of male *P. xylostella*. Left lateral cuticle is excluded between ventral and dorsal midline to expose structures *in situ*. Roman numerals denote abdominal segments; bar = 500 µm.

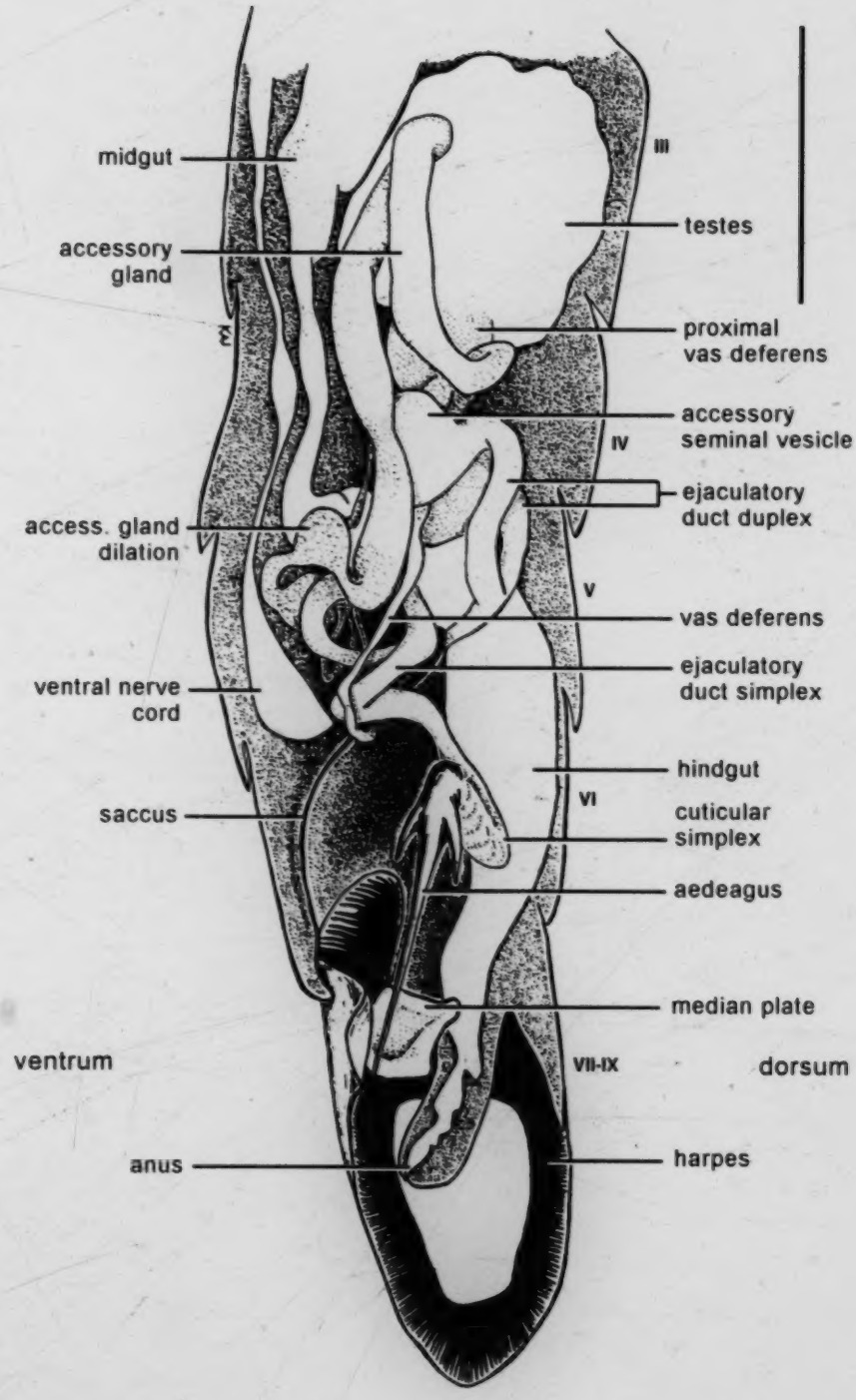
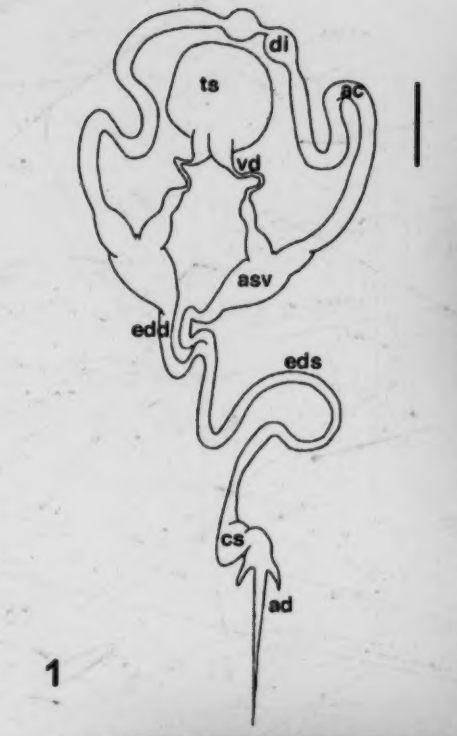
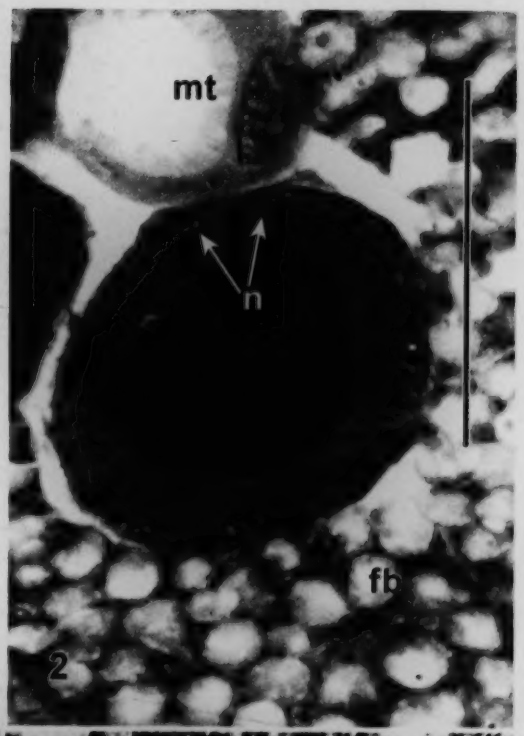

midgut
accessory gland
III
testes
proximal vas deferens
accessory seminal vesicle
IV
ejaculatory duct duplex
access. gland dilation
V
vas deferens
ejaculatory duct simplex
ventral nerve cord
hindgut
VI
saccus
cuticular simplex
aedeagus
median plate
ventrum
VII-IX
dorsum
anus
harpes

Plate 2-IV. 1) Line drawing of male reproductive structures, bar = 200 μm; 2) Transverse section through male accessory gland, bar = 50 μm; 3) Transverse section through ejaculatory duct simplex showing differences between anterior (an) and posterior sections (ps) of duct, bar = 50 μm; 4) Sagittal section through cuticular simplex showing both anterior (an) and posterior (ps) lumen, bar = 50 μm. Abbreviations: **ac** - accessory gland, **ad** - aedeagus, **an** - anterior, **asv** - accessory seminal vesicle, **cs** - cuticular simplex, **di** - dilation of accessory gland, **edd** - ejaculatory duct duplex, **eds** - ejaculatory duct simplex, **fb** - fat body, **hp** - hair pencils, **mt** - Malpighian tubule, **mu** - muscle, **n** - cell nucleus, **ps** - posterior, **ts** - testes, **vd** - vas deferens.



1



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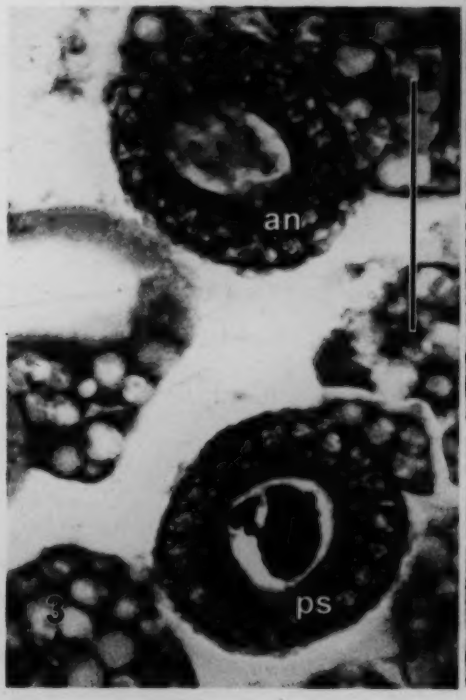

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Plate 2-V. Pre-copulatory behaviors of male moth; female is out of focus in background: 1) arrival of male facing female, 2) wing fanning and extrusion of hair pencils at abdominal tip, 3) orienting genitalia to female, 4) contacting female, 5) cessation of wing fanning, 6) *in copula*; female above, male below.

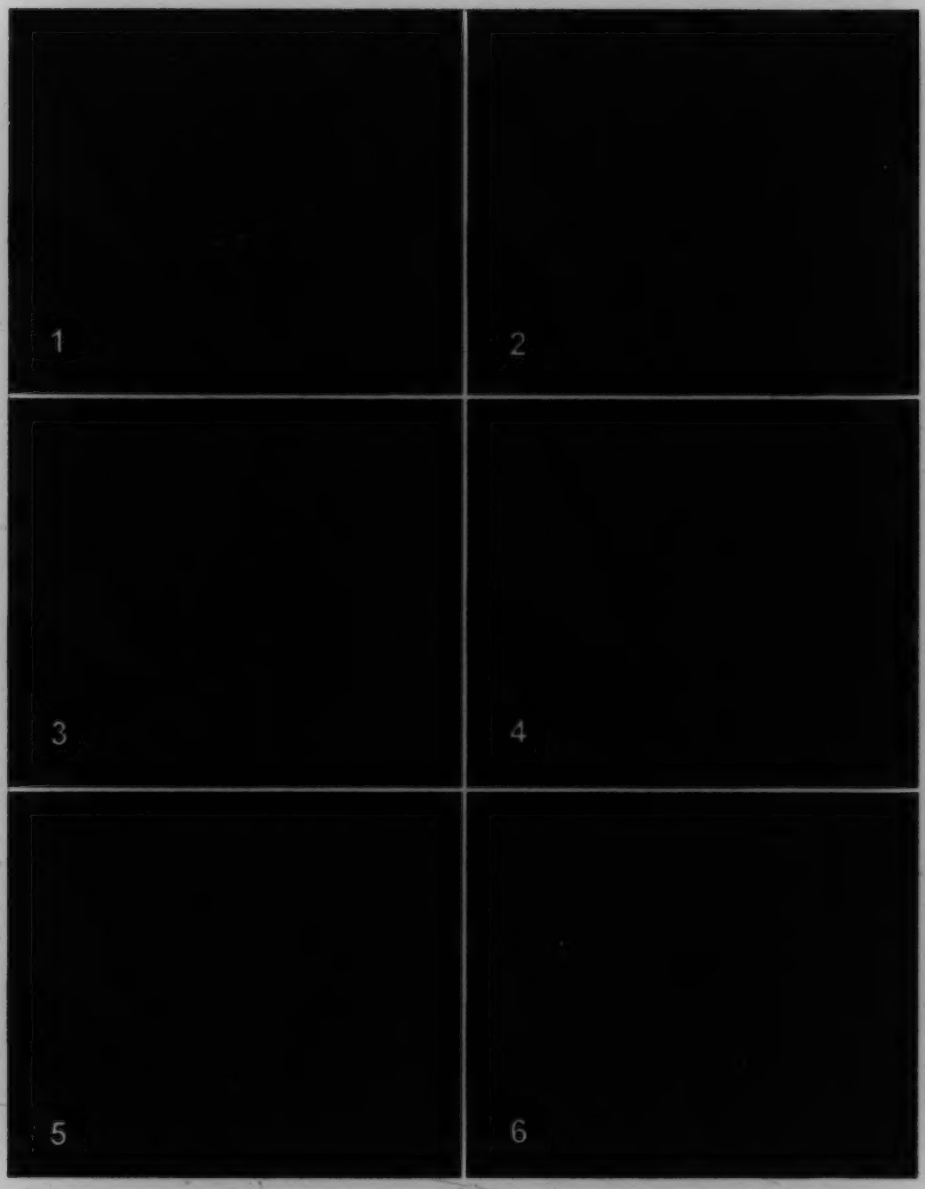
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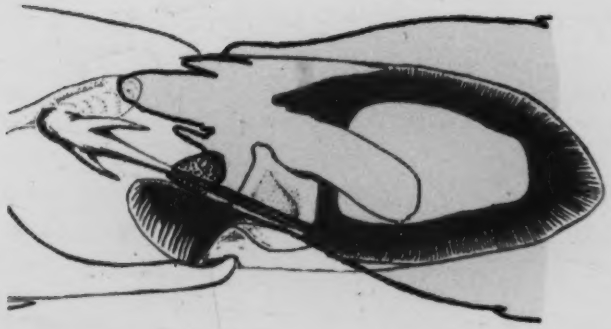
Plate 2-VI. Compatibility of genitalia: 1) overlapping outlines of genitalia from reconstructions, male on left, female on right; scanning electron micrographs of 2) tip of male abdomen, posterior oblique-perpendicular view; 3) tip of female abdomen, ventral view; 4) tip of male abdomen, ventral view; 5) tip of female abdomen, posterior perpendicular view. Bars = 100 μm. Colors represent overlapping structures during copulation.



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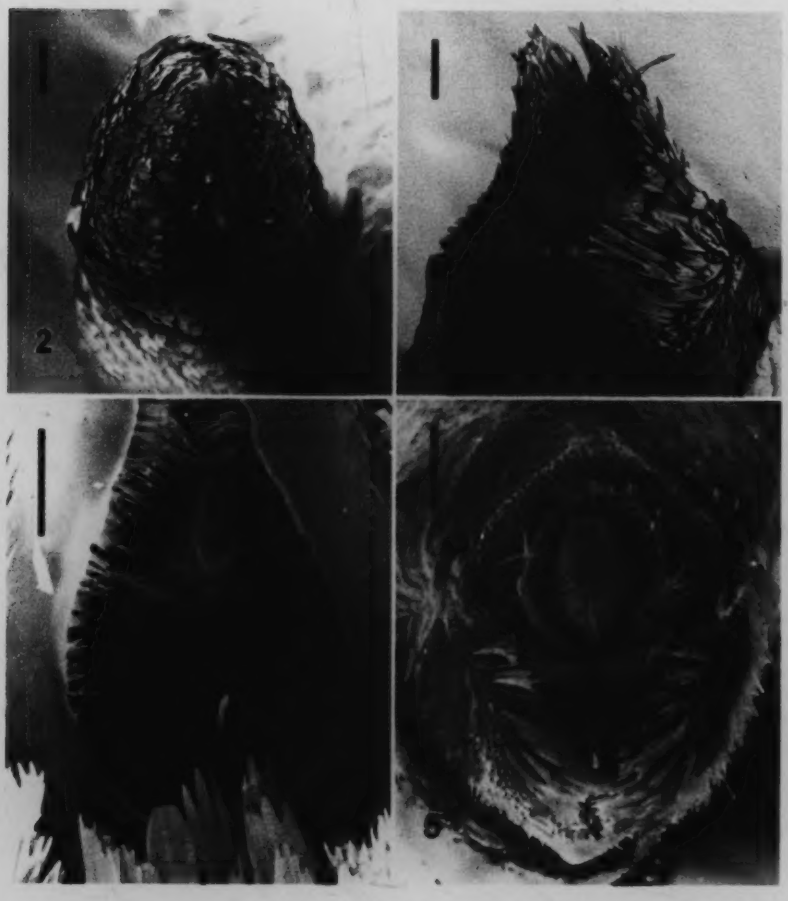

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Plate 2-VII. Scanning electron micrographs of ostium bursa exhibiting effects of mating; 1) virgin female, bar = 3 μm; 2) mated female, bar = 5 μm.

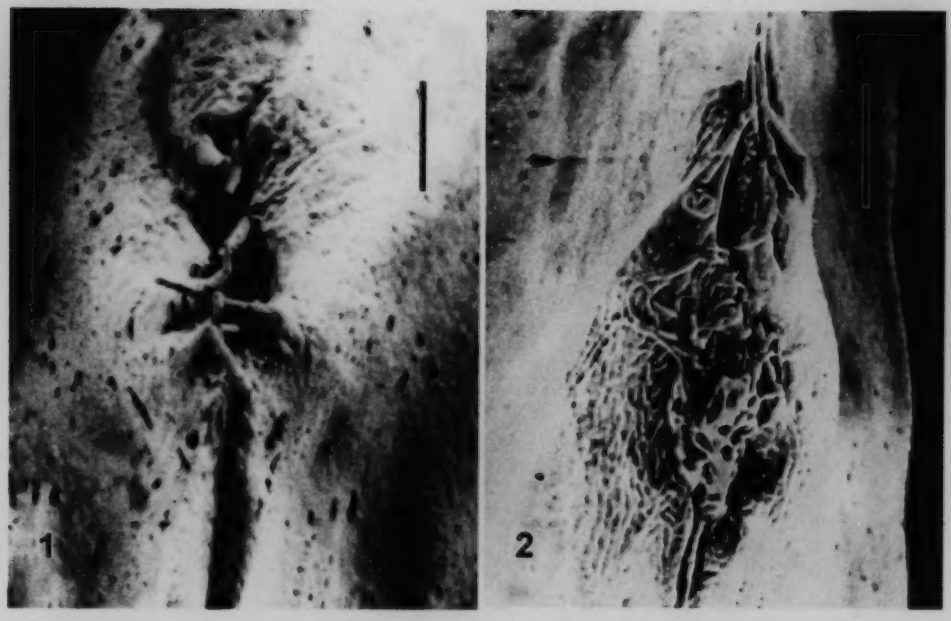
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Plate 2-VIII. Scanning electron micrographs of sample aedeagi from seven populations: 1) Australia, 2) Canada, 3) China, 4) Florida, 5) Netherlands, 6) New York, and 7) Switzerland; bar = 100 μm.

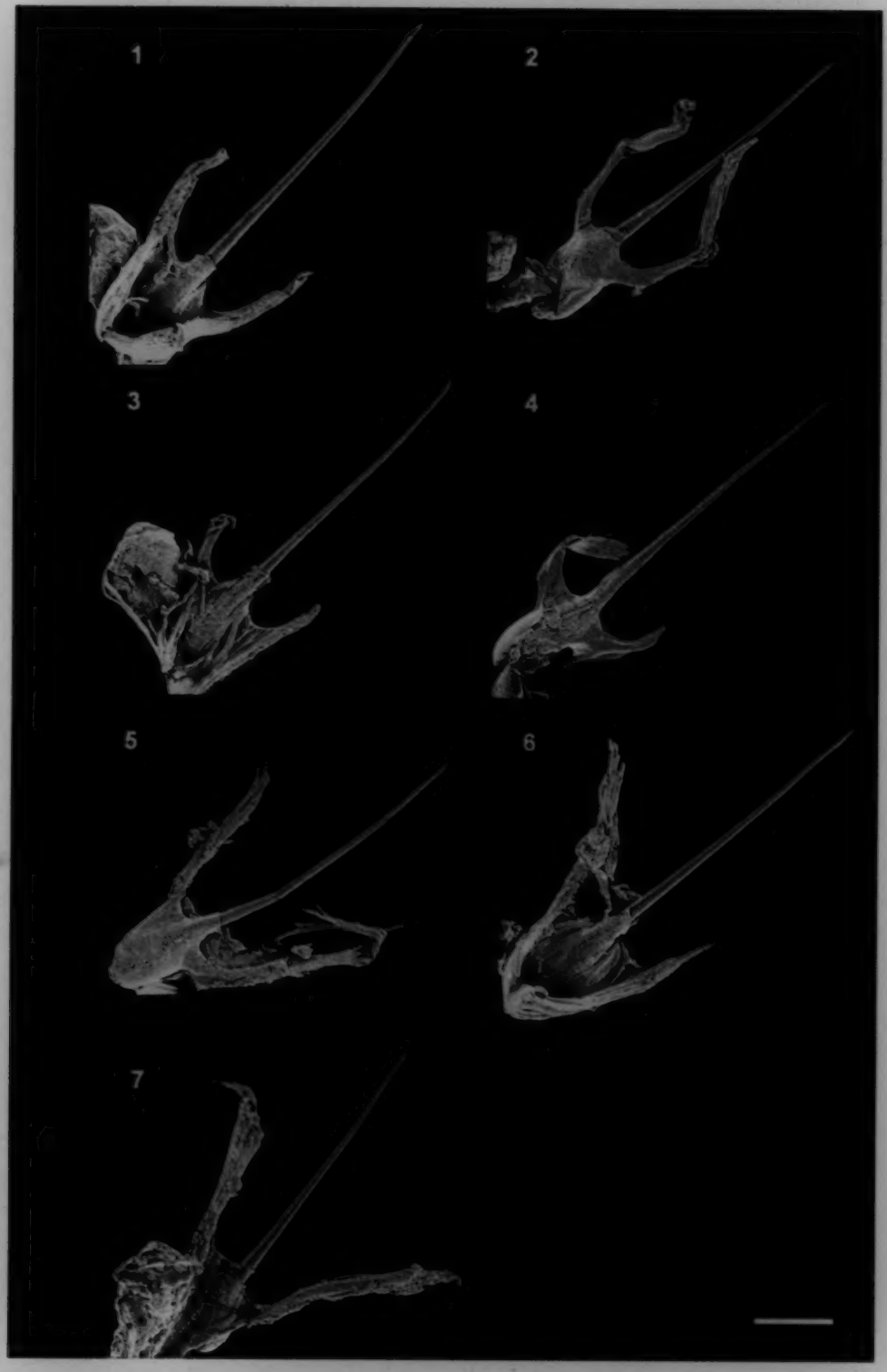
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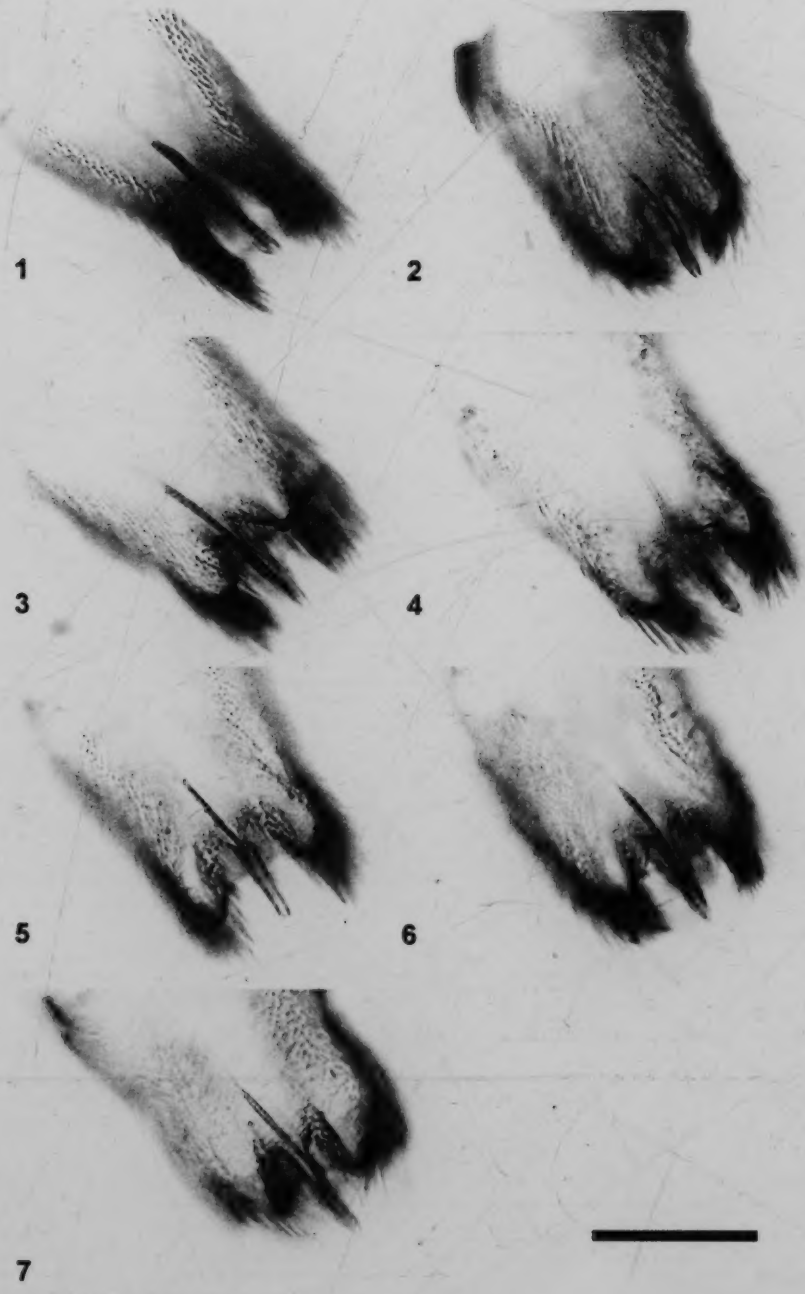
Plate 2-IX. Light micrographs of sample bursal ducts from seven populations: 1) Australia, 2) Canada, 3) China, 4) Florida, 5) Netherlands, 6) New York, and 7) Switzerland; bar = 400 μm.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Chapter 3:

Oviposition site selection by *Plutella xylostella* [‡]

3.1 Introduction

A number of investigators have examined the attractiveness of secondary compounds of crucifers (*i.e.* glucosinolates and volatile mustard oils) to ovipositing diamondback moths (Renwick and Radke, 1990; Reed *et al.*, 1989; Gupta and Thorsteinson, 1960). However, details about the nature of such attractants and their importance to ovipositing *P. xylostella* are virtually unknown. In contrast, investigations of other cruciferous pests, such as *D. radicum* and *P. rapae*, have demonstrated factors involved in host plant location (Nottingham, 1988; Renwick and Radke, 1988) and stimuli critical for host acceptance and associated oviposition behaviors (Klijnstra, 1985).

Because chemical and microbial controls are no longer effective against some populations of diamondback moth (see Talekar and Shelton, 1993), alternative control strategies are urgently required. Just as life history knowledge is crucial for development of successful integrated pest management strategies, behavioral information is also essential for understanding plant-insect interactions (Opp and Prokopy, 1986). Such information could be crucial for the development of novel control strategies of this moth.

This study of mechanisms of host selection by *P. xylostella* was conducted to determine how oviposition behavior is regulated by environmental features (such as chemical and mechanical stimuli from the host plant).

[‡] A version of this chapter has been published. Justus, K.A. and Mitchell, B.K. 1996. Journal of Insect Behavior 9: 887-898.

3.2 Materials and Methods

3.2.1 Insects

(See Chapter 2 for description of colony rearing.)

Pupae were held singly in corked glass vials (8 cm x 2.5 cm diameter) until emergence. Newly emerged adults were sexed and placed in a glass cage (12.7 cm x 17.3 cm x 29.6 cm) as a group (4-8) in an approximate 50:50 (male:female) ratio. Honey-water and a freshly cut stem of *B. napus* in water were also placed in this 'mating cage'. Moths were allowed to interact freely for a three-day period. On the fourth day, females were removed from the mating cage and placed into individual oviposition arenas (8 cm x 2.5 cm diameter glass vials) that contained one of the treated substrates.

3.2.2 Oviposition arenas

'Lunar blue' 60 lb bond paper (Smead®) was cut into strips 1.2 m x 10 cm. Prior to each experiment, a 1.2 cm x 2.0 cm area at one end of the strip was treated in one of the following ways: painted with deionized H_20 and allowed to dry (H20), painted with 0.5 mM sinigrin (Aldrich Chem. Co.) dissolved in H_20 and allowed to dry (SIN), or rubbed with a squashed *B. napus* leaf (NAP). Grooves (GRV) to imitate leaf veins were made in the paper (at the treated area) using the blunt edge of a one-sided razor blade. The treated strip was then placed vertically in an oviposition arena containing a female moth, and held in place by a cork. For assays involving host volatiles only (VOL), vials were sealed with fibreglass screening (1 mm mesh) and held approximately 3 cm above fresh *B. napus* homogenate so that odors should have been detectable but contact with the homogenate impossible. (See Table 3-II for combinations of treatments and sample sizes.)

3.2.3 Observations

A single moth was introduced to an oviposition arena at the onset of the scotophase, and observed under a red safe light (General Electric model 810-S, Kodak filter series 1A). Continuous focal sampling was recorded on audiocassette tapes, and the information transferred to Observer 2.0 software (Noldus Information Technology, the Netherlands). Locations and numbers of eggs were noted during the observation period and immediately following the 8 hr scotophase.

3.2.4 Data analysis

First-order transition sequences were illustrated in transition matrices (preceding behavior x succeeding behavior) for each moth, and G-tests used to test for similarities between moths. Cells on the descending diagonal of matrices held structural zeroes since a behavior could not be followed by itself (*i.e.* considered to be a continuation of the behavior (Slater, 1973)). Expected values of behavior transitions were calculated using Stephen-Denning iterative proportional fitting (Jobson, 1992). Observed and expected transitions were tested for significance using Chi-square goodness of fit. Kruskal-Wallis non-parametric analysis of variance was used for egg count and behavior duration data.

3.3 Results

Descriptions of behaviors are given in Table 3-I and selected behaviors are depicted in Figure 3-1. Antennal rotation (AR) began almost immediately after placing the moth in an oviposition arena. Walking, grooming, extending the proboscis, standing still, and AR were the only behaviors observed prior to the onset of the scotophase. Advanced behaviors only took place at or near the treated part of the paper strip.

Rates at which the antennae struck the substrate during antennation were relatively constant (2.5 ± 0.3 mean strikes per second ± standard deviation) for all moths regardless of substrate. Rate of ovipositor sweeps (OS) was slower when OS led to egg deposition (OV) (Fig. 3-2), which was attributed to a decrease in OS rate just prior to OV (Fig. 3-3). This decrease in OS rate, combined with cessation of walking, was labelled the OS determinant phase (OS^{dt}); the quicker, preceeding OS phase was labelled the OS search phase (OS^{sr}). Decrease in OS rate was not observed when OS did not lead to OV.

First-order behavior transitions did not differ significantly among moths that oviposited, and were combined in one matrix. Observed behavior transitions differed significantly from expected (Chi square; $\alpha = 0.05$, $P < 0.0001$) (Fig. 3-4). Probabilities of behavior transitions were calculated from 27 ovipositing moths and are given in a kinematic diagram (Fig. 3-5). Ovipositing moths followed a linear sequence (AR → AN → OS^{sr} → OS^{dt} → OV) but did not necessarily complete each attempt. Also, moths could bypass AN and enter OS directly from AR only if they had already performed AN but had not yet entered OV or "Other" states. Any OV attempt (defined by the initiation of AN) could be aborted, in which case a moth would return to a previous behavior. Usually, the sequence was not aborted once OS^{dt} occurred (Fig. 3-5). A complete oviposition sequence resulted in the deposition of one egg. Only 13.6% of oviposition attempts resulted in egg deposition.

Behaviors performed were somewhat dependent on substrate treatment (Table 3-II) and time between oviposition events was highly variable (range 73.8 - 1068.1 seconds). However, when each oviposition event was partitioned into durations of AR, AN, and OS, moths given substrates that included volatiles (*i.e.* SIN+VOL, NAP, and

NAP+GRV) spent significantly less time performing AR than moths given non-volatile substrates (*i.e.* SIN and SIN+GRV) (Kruskal-Wallis, $\alpha = 0.05$, $P < 0.0001$) (Table 3-III). (Note that H20+VOL was not included since this did not result in oviposition.) Time spent performing AN and OS did not differ among moths regardless of substrate treatment (Table 3-III).

Total number of eggs deposited was not significantly different among treatments for moths that oviposited. However, a proportionately greater number of eggs was deposited in the first hour of the scotophase when moths were given both volatile and contact stimuli (Table 3-IV).

3.4 Discussion

Regardless of substrate, first-order transition probabilities were similar among all moths that oviposited. As well, transition probabilities differed from random, suggesting that oviposition is a process characterized by a structured sequence of events. Three of the seven behaviors identified in ovipositing moths (PE, GR, and ST), were considered ancillary behaviors because they were not observed in all ovipositing moths, did not occur with any regular periodicity, and have also been observed in males and virgin females (personal observation). *Plutella xylostella* exhibits four oviposition behaviors that appear to be organized as a linear sequence, with each subsequent behavior depending on the previous. However, this sequence is probabilistic rather than deterministic because the probability of a complete sequence is less than 100%. Although there appears to be no regular periodicity to egg deposition, several external

factors influence ovipositing *P. xylostella* , and the probability of oviposition depends to some degree on the perception of those factors.

AR is likely involved in detection of host volatiles because of the presence of multiporous sensilla on the antennae (Chapter 5; Chow *et al.*, 1984). Such movements by antennae allow moths to sample larger volumes of air and create wind vortices around the antennae, both of which would facilitate sensillar contact with odorant molecules. AR is analogous to sniffing in mammals and antennule flicking in lobsters; although this does not presume contact with a receptive surface, it should increase that probability. Palaniswamy *et al.* (1986) noted that both male and female *P. xylostella* respond to host plant volatiles, and Gupta and Thorsteinson (1960) reported that allyl isothiocyanate (the volatile produced when sinigrin is hydrolyzed) enhanced oviposition. This study showed that moths given both volatile and contact stimuli deposit significantly more eggs during the first hour of the scotophase, than those without volatiles. As well, moths spent significantly more time involved in AR when volatiles were absent than when volatiles were present. AR is likely involved in host location, but it may be of secondary importance in such small enclosures because moths will explore and oviposit even in the absence of olfactory cues.

Presumably, AR has some effect on AN because moths not given volatiles spend a larger proportion of time in AR before making the transition to AN. The precise nature of the relationship between AR and AN is not understood and requires further study; some threshold of volatile may trigger the switch to AN, although it can occur in spite of a lack of olfactory input.

AN is likely the first mechanism by which moths 'taste' a potential oviposition substrate via uniporous sensilla present on the distal segments of the antennae (Chapter 6; Chow, *et al.*, 1984). Faucheux (1991) described uniporous sensilla on the antennae of *Homoeosoma nebulella* (Lepidoptera: Pyralidae), as did Cuperus (1986) for *Yponomeuta vigintipunctatus*, *Y. cagnagellus* (Yponomeutidae) and *Adoxophyes orana* (Tortricidae) but neither noted whether antennae were involved in oviposition behavior. Conversely, Chadha and Roome (1980) observed similar AN behavior in *Chilo partellus* (Lepidoptera: Pyralidae), but did not describe antennal sensilla.

AN, which may be analogous to tarsal drumming in *Pieris* spp., appears to be a prerequisite for OS, because OS occurs only following AN. It seems likely that *P. xylostella* proceeds from AN to OS if chemical stimuli are positive/sufficient, and returns to AR if chemical stimuli are negative/insufficient.

OS appears to be divided into a 'search phase' and a 'determinant phase'. The search phase is characterized by a relatively fast rate of sweeping, and is the period in which gustatory and tactile information seem to be most important in oviposition site selection. It is during the OS search phase that moths may abort the sequence and begin again at either AR or AN. Four to six uniporous sensilla are present on each lobe of the ovipositor, and are surrounded by many long, aporous sensilla (Chapter 6) that are likely mechanosensory in function. Thus, OS likely facilitates both chemical and mechanical input involved in locating an egg deposition site, and these two mechanisms probably act in tandem.

Although contact chemosensilla on the ovipositor may initially seem to be redundant to those of the antennae, sensilla of the antennae and ovipositor might respond

to different stimuli. There is some speculation in the literature that antennal taste sensilla, in general, detect contact pheromones used for conspecific identification. However, if these receptors also respond to host plant chemicals, OS may act as a 'safety check' for depositing an egg at a specific site. Since there is a relatively large distance between antennal tip and ovipositor, the antennae may identify a site that is not contiguous to the site of egg deposition. Egg location is important for larval survival because first instar larvae are leaf miners and dessicate very quickly if prevented from burrowing under the protective plant cuticle (personal observation).

Chilo partellus and *Ostrinia nubilalis* (Lepidoptera: Pyralidae) have similar arrangements of contact chemo- and mechanosensilla (Chadha and Roome, 1980; Marion-Poll *et al.*, 1992) which they appear to use for detecting and selecting an oviposition site. In particular, mechanosensilla are used for arranging eggs in orderly rows. Because *P. xylostella* do not lay eggs in batches and usually deposit eggs in crevices, the aporous sensilla are presumably the means by which such crevices are located. Crevices may offer protection to eggs, or perhaps allow larvae easier access to parenchymatous tissues. The initial hypothesis that an absence of grooves in the substrate would lead to a higher proportion of time spent performing OS was incorrect; while mechanosensory hairs likely facilitate the perception grooves and grooves are preferred locations for egg deposition, they are not a required stimulus for egg release but instead are involved in egg placement.

The OS determinant phase is characterized by a marked decrease in OS rate and by the cessation of walking. Once this phase begins, it nearly always leads to egg deposition. Because OS^{dt} and OV are so closely linked, it is likely that OS^{dt} facilitates

abdominal muscle contractions and the movement of the egg through the ovipositor, as in *Bombyx mori* (Yamaoka *et al.*, 1971).

Renwick and Radke (1990) demonstrated that glucosinolates are important to ovipositing *P. xylostella*. The most important influence on host selection by *P. xylostella*, appears to be the presence of contact stimuli that may be perceived via contact chemosensilla on the antennae, tarsi, and ovipositor, but whether chemosensilla from all three body parts respond to identical stimuli is unknown. Host plant volatiles are likely involved in host location as discussed by Palaniswamy *et al.* (1986), and also enhance egg deposition rate. The presence of grooves does not appear to induce oviposition by *P. xylostella*, but does influence egg placement.

Table 3-I. Ethogram: behaviors performed by ovipositing *P. xylostella*

*Still (ST):	no visible movement. Moth is standing. Antennae are forward. Posterior end of wings nearly touch the substrate (Fig. 3-1a).
Antennal rotation (AR):	antennae are independently rotated posteriorly, laterally, and then anteriorly to the resting position. Antennae may rotate in unison or separately, not necessarily alternating. The arc of rotation varies greatly within individuals (Fig. 3-1b & c). AR can occur while insect is walking or standing.
Antennation (AN):	antennae simultaneously and repeatedly strike the surface of the substrate with the most distal segment(s). Angle of the antennae decreases with respect to the head via muscles of the scape and pedicel, and angle of the head with respect to the body diminished by bending at the head-thorax junction (Fig. 3-1d). Usually occurs while insect is walking, but can occur while standing.
Ovipositor sweep (OS):	Distal abdominal segments bend, lifting the wings and extruding the ovipositor. Ovipositor touches substrate at a point lateral to the longitudinal axis of the moth (Fig. 3-1e). Ovipositor is swept mediad from the lateral point (Fig. 3-1f & g). Sweep usually alternates sides, but can occur on the same side twice before changing sides. OS occurs while insect is walking, but can continue if walking ceases (see results Fig. 3-3). OS occurs at relatively fast rate while moth is walking (termed OS search phase) and slows after walking ceases (termed OS determinant phase).
Oviposition (OV):	A single egg is discharged through the ovipositor as the abdomen moves side to side slightly. Egg is glued on its side to the substrate.
*Proboscis extension (PE):	proboscis is extended to touch substrate. (Not done by all individuals; can occur in conjunction with AR).
*Grooming (GR):	foreleg is placed at base of pedicel of antenna and slid down the length of antenna in a combing fashion. Moth is standing.

* These behaviors are grouped as "Other" in the kinematic graph.

Table 3-II. Behaviors and egg placement by *P. xylostella* presented with treated substrates in oviposition arenas.

Treatment	n	Behaviors completed	Egg placement
H2O	4	AR & ST	no eggs
H20+VOL	5	AR, AN, & other*	no eggs
SIN	4	AR, AN, OS, OV, & other*	100% on substrate
SIN+GRV	5	AR, AN, OS, OV, & other*	96% on substrate 62% in grooves
SIN+VOL	4	AR, AN, OS, OV, & other*	92% on substrate
NAP	7	AR, AN, OS, OV, & other*	93% on substrate
NAP+GRV	7	AR, AN, OS, OV, & other*	95% on substrate 85% in grooves

* "others" are ST, PE, and GR, but not all moths exhibited these behaviors.

Table 3-III. Time spent performing antennal rotation (AR), antennation (AN), and ovipositor sweeping (OS) during individual oviposition sequences (expressed as the range of % total time for each oviposition event) for moths given substrates treated with sinigrin (SIN), plant juice (NAP), volatiles (VOL), and grooves (GRV).

Substrate	n	%AR	%AN	%OS
SIN	4	63.9-79.9[a]	2.7-6.3	7.9-67.6
SIN+GRV	5	62.1-80.4[a]	5.5-12.9	5.6-23.7
SIN+VOL	4	27.2-54.1[b]	7.0-27.2	4.1-40.0
NAP	7	29.6-55.2[b]	2.2-31.3	15.3-73.6
NAP+GRV	7	21.9-57.9[b]	2.1-24.6	26.8-75.7

[a,b] Kruskal-Wallis; $\alpha = 0.05$, $P < 0.0001$.

Table 3-IV. Oviposition rate: number of eggs oviposited during the first hour of the scotophase and number of eggs oviposited during entire (8 hr) scotophase.

Substrate	n	mean number of eggs first hour (± s.d.)	mean number of eggs scotophase (± s.d.)
SIN	4	3.25 ± 0.5[a]	27.25 ± 6.7
SIN+GRV	5	5.20 ± 1.9[a]	24.40 ± 7.6
SIN+VOL	4	11.00 ± 5.4[b]	26.00 ± 7.5
NAP	7	18.40 ± 10.3[b]	27.80 ± 17.4
NAP+GRV	7	14.70 ± 7.9[b]	31.30 ± 15.5

[a,b] Kruskal-Wallis; $\alpha = 0.05$; $P < 0.001$.

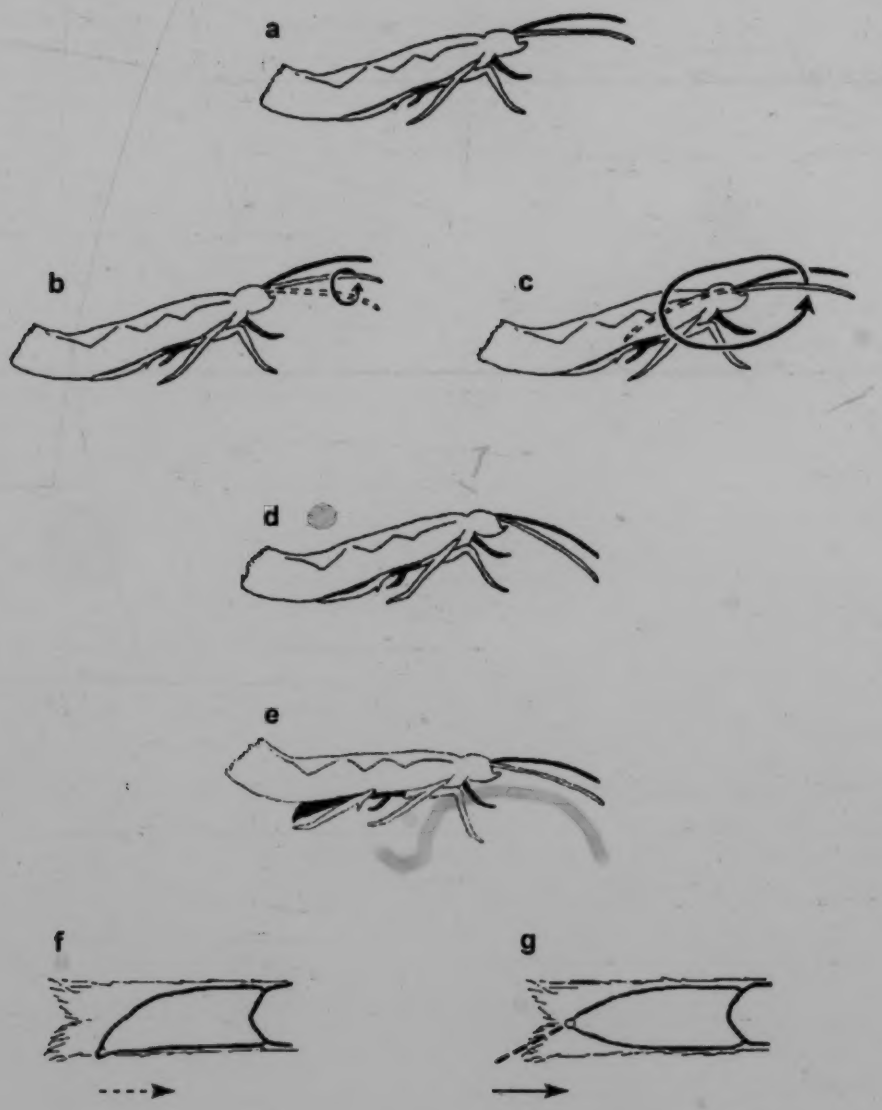

Figure 3-1. Oviposition behaviors of *P. xylostella*: a) resting position; b) antennal rotation - acute arc; c) antennal rotation - obtuse arc; d) antennation - head is lowered and antennae strike surface of substrate; e) ovipositor sweep – note position of abdomen; f) ovipositor sweep (ventral view), starting point of one sweep; dashed arrow = direction and distance moth will travel; g) ovipositor sweep (ventral view), end point of sweep; dashed line = ovipositor dragged on substrate; arrow = direction and distance travelled by moth.

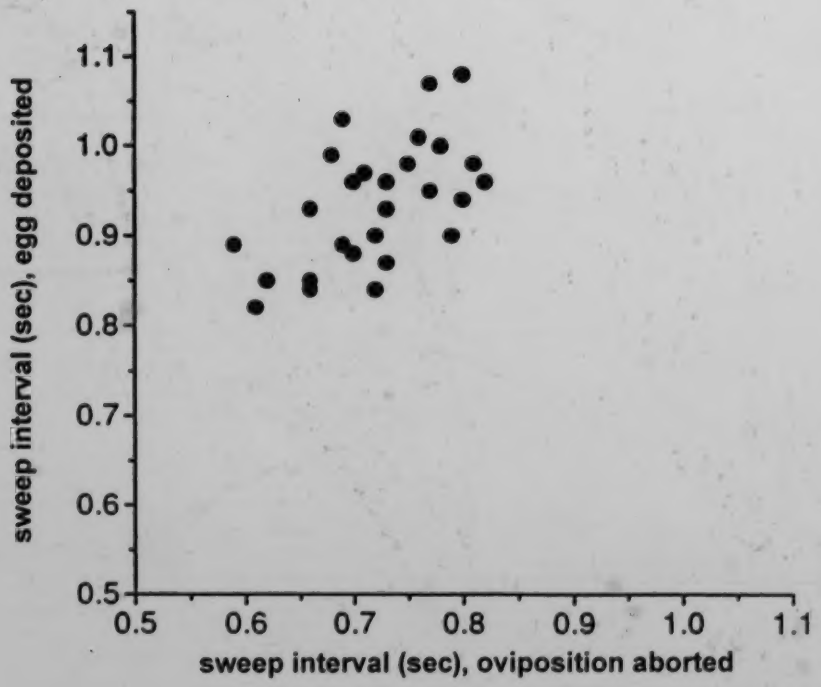

Figure 3-2. Ovipositor sweep intervals of 27 moths. Each data point represents the mean sweep interval when egg deposition occurred (y-axis) and the mean sweep interval when the oviposition sequence was aborted (x-axis), of each moth.

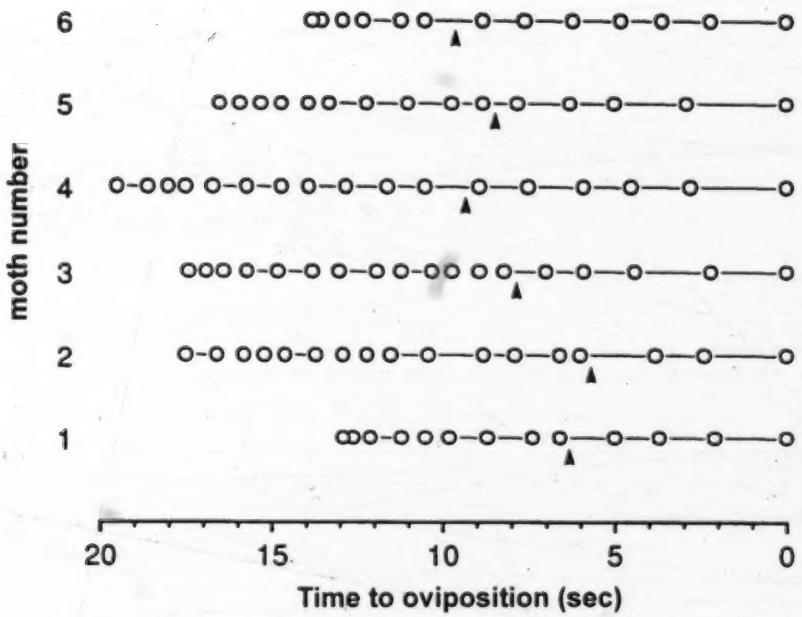

Figure 3-3. Diagrammatic representation of OS intervals prior to one oviposition event by six moths. Time "0" is egg deposition; "o" represents one sweep; arrows indicate cessation of walking.

Observed:

	AR	AN	OS	OV	TTL
AR	---	519	278	0	797
AN	388	---	301	0	689
OS	267	170	---	142	579
OV	129	0	0	---	129
TTL	784	689	579	142	2194

Expected:

	AR	AN	OS	OV	TTL
AR	---	418.2	317.1	61.7	797
AN	407.8	---	235.4	45.9	689.1
OS	309	235.3	---	34.7	579
OS	55.1	42	31.9	---	129
TTL	771.9	695.5	584.4	142.3	2194.1

Figure 3-4. Behavior transition matrices. Expected transitions calculated via Stephen-Denning interative proportional fitting (see Jobson, 1992).

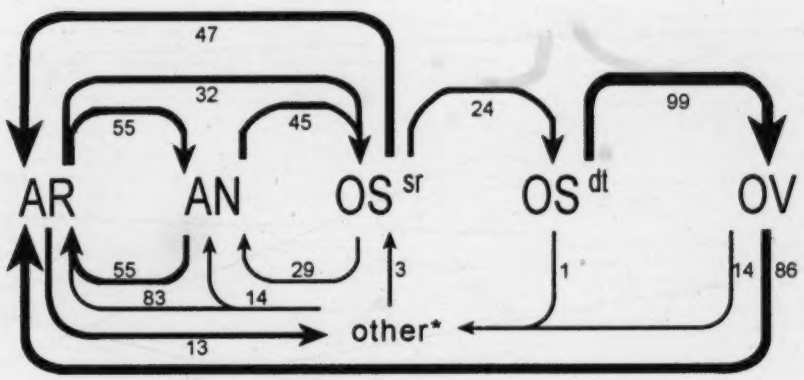

Figure 3-5. Kinematic diagram (n = 27 moths). Thickness of lines represent likelihood of occurrence. Numbers are percent probability of transition. **AR** - antennal rotation; **AN** - antennation; **OS^{sr}** - ovipositor sweep search phase; **OS^{dt}** - ovipositor sweep determinant phase; **OV** - oviposition. Other* includes grooming, proboscis extension, and still. (See Table 3-I for behavior definitions.)

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Chapter 4:

Characteristics of the phylloplane influence oviposition by *Plutella xylostella*

4.1 Introduction

Waxiness of crucifer leaf surfaces affects larval behaviors of insect pests of *Brassica* spp. Glossy varieties of *B. oleracea* support lower populations of the cabbage aphid, *Brevicoryne brassicae* (Homoptera: Aphididae) (Thompson, 1963), and crucifer flea beetles, *P. cruciferae*, feed less on waxy plants and in uncharacteristic patterns (Bodnaryk, 1992). Neonate *P. xylostella* larvae move more rapidly and spend more time searching on surfaces with reduced wax (Eigenbrode *et al.*, 1991a, 1991b). Eigenbrode and Shelton (1992) concluded that "reduction in waxes is the basis for resistance in genetically glossy strains" with *P. xylostella* mortality occurring predominantly during the first instar (Eigenbrode *et al.*, 1991b). Proposed mechanisms for these effects are mainly mechanical, such as increased traction of predators on reduced waxes (Eigenbrode *et al.*, 1996; Shah, 1982), and prolonged exposure associated with longer search periods of neonate larvae (Eigenbrode *et al.*, 1991b).

Leaf waxes can also affect oviposition but mechanisms are unknown. Rivet and Albert (1990) found that ovipositing spruce budworm, *C. fumiferana*, require leaf waxes for host detection and suggested that gustation is involved in the detection process. For cruciferous insects, reduced waxes increased ovipositon by the cabbage maggot, *D. radicum* (Prokopy *et al.*, 1983) and by *P. xylostella* (Uematsu and Sakanoshita, 1989).

Prokopy *et al.* (1983) suggested that visual cues are important because waxy leaves reflect different spectra than glossy leaves; Uematsu and Sakanoshita (1989) stated that waxes are inhibitory to *P. xylostella* oviposition but did not propose possible mechanisms.

Few investigations of surface wax effects on oviposition by *P. xylostella* have been conducted, and the value of previous research is diminished by the use of procedures that did not adequately account for all possible influences at the leaf surface. For example, studies using high glucosinolate *B. oleracea* varieties (*e.g.* cabbage, broccoli, cauliflower) may mask effects of epicuticular waxes if glucosinolates stimulate the behavior in question, and comparisons of waxy and glossy species (*e.g. B. juncea* vs. *B. oleracea*) do not account for differences in leaf morphology (*e.g.* presence/absence of trichomes) or chemical compositions (*e.g.* differences in glucosinolate content).

The objective of this study was to investigate the isolated effects of host plant epicuticular wax content on oviposition site selection by *P. xylostella.* Because the phylloplane inherently possesses numerous variables that cannot be measured in any single assay, this study used three radically different approaches. The first compared oviposition on untreated control plants and on plants that had epicuticular wax reduced by application of the herbicide, *S*-ethyl dipropylthiocarbamate (EPTC). Carbamate herbicides limit fatty acid elongation, and EPTC selectively inhibits development of very long chain (> C18) fatty acids of leaf surface lipids (see Gronwald, 1991). The second approach compared oviposition on two strains of *B. napus* with markedly different wax blooms. The third approach compared oviposition by *P. xylostella* when exposed to parafilm that had been applied to leaves of the two strains of *B. napus* for transfer of leaf

surface components. Mechanisms that could explain the importance of phylloplane characteristics on oviposition were identified and discussed.

4.2 Materials and Methods

(See Chapter 2 for description of colony rearing.)

4.2.1 Herbicide assays

Potted *B. napus* cv. Westar was grown in a glasshouse (22°C, 16L:8D), watered daily and fertilized (20:20:20) weekly. Each week, 20 ml of 10^{-3} M EPTC (Appendix I-4) was added to soil at the bases of experimental plants and 20 ml of deionized H_20 was added to control plants.

Approximately three weeks after seeding (growth stage 2.6 - 3.1 of Harper and Berkenkamp, 1975), two treated and two control plants were set in 60 cm^3 cages in alternate fashion so that plants receiving the same treatment were diagonally opposite each other. Twenty newly emerged moths (10 male,10 female) were added to each cage. Honey-water was provided. After seven nights, plants were removed and eggs on each plant were counted. This was repeated six times. Because EPTC-treated plants showed less vigor and poor health beyond four weeks of age, this procedure could not be repeated on plants at later developmental stages.

Abaxial and adaxial surfaces of sample leaves of both EPTC-treated and untreated plants were prepared using an Emitech K1250 cryopreparation system (Appendix III-2) and examined with a JSM-6301FXV field emission scanning electron microscope.

4.2.2 Phenotypic assays

Studies were conducted in field plots at the Alberta Research Council, Vegreville. Plots were on land that was fallow during the year prior to seeding, and had soil characterized as black chernozemic. Each year the soil was fertilized according to soil test requirements for canola production (Thomas, 1990).

Seed used in this study was obtained from a parental waxy strain of *B. napus* which produced two types of (S1) progeny: glossy (dark green with reduced surface wax) and waxy (lighter in color with normal wax bloom). In 1995, S1 seed was used, and harvested seed (S2) from plants not used in field assays was used in 1996 assays. Because plants were open-pollinated in 1995, fewer plants of the glossy phenotype were available for germination in 1996, necessitating slight modification in experimental design (see below).

All seed was uniformly coated with Vitavax fungicide prior to seeding to reduce seedling mortality by phytopathogens. A 6 m x 12 m plot was seeded (1.88 g/6 m row) in alternate rows of glossy and waxy phenotypes (Fig. 4-1). Seeding was performed with a coulter double disc press drill at a depth of 1 cm with a 50 cm distance between rows.

In 1995, glossy and waxy plants at rosette, early flowering, and seed pod development (growth stages 3.1, 4.1, and 4.3, respectively, of Harper and Berkenkamp, 1975) were assessed for susceptibilities to oviposition by *P. xylostella*. Pyramidal cages measuring 1 m x 1 m at the base (see Dosdall *et al.*, 1996) were placed over plants in one row of the glossy and one of the waxy phenotypes enclosing 40 - 50 plants of each. Twenty newly emerged adult *P. xylostella* (10 male, 10 female) were placed in each cage. Four cages were used per plant stage, and were set randomly within the plot. After

seven nights, moths were removed and counted, plants were uprooted, and five randomly chosen plants of each phenotype were examined for condition (*e.g.* leaf senescence), number of leaves present, height and basal diameter of stem, number and placement of eggs, and presence of other insects/diseases.

Similar procedures were used in 1996, with two exceptions. First, only rosette and early flowering plants (growth stages 3.1 and 4.1, respectively) were evaluated. Second, alternate plantings of rows of glossy and waxy seed resulted in rows of mixed phenotypes (likely due to open-pollination in 1995). Therefore, prior to each cage placement, rows were thinned so that cages enclosed one row of 10 glossy plants and one row of 10 waxy plants. Five cages were used for each plant stage.

Nested ANOVAs were performed separately on 1995 and 1996 data because of uneven sample sizes (3 stages x 4 cages in 1995; 2 stages x 5 cages in 1996) and because of a dilution effect in 1995 with more plants per cage. Year data were normalized by dividing number of eggs on glossy plants by total number of eggs on both plant types, and these proportions compared with a paired t-test. Normalized egg counts were also tested for egg location on plants with a Kruskal-Wallis test.

Abaxial and adaxial surfaces of sample leaves of both glossy and waxy phenotypes were prepared using an Emitech K1250 cryopreparation system (Appendix III-2) and examined with a JSM-6301FXV field emission scanning electron microscope. For reference, leaves of *S. alba*, a non-preferred host of ovipositing *P. xylostella* (personal observation; Talekar and Shelton, 1993), were also examined using the same method.

4.2.3 Parafilm assays

Parafilm was used to transfer oviposition stimulants from canola leaves using a technique modified from Papaj and Prokopy (1986). Immediately preceding its use in bioassays, a 2 cm x 2 cm square of Parafilm 'M' ® (American Can Company, USA) was placed on the adaxial surface of a leaf of *B. napus* (cv. Westar, S2 glossy, or S2 waxy), and covered with a slightly larger piece of filter paper. A second piece of filter paper was placed under the leaf, and these four layers were sandwiched between two small pieces of plexiglass. Gentle pressure, so as not to damage the leaf, was applied for 30 seconds. The parafilm was then lifted from the leaf surface and stretched over the mouth of a glass shell vial. Untreated parafilm squares were not pressed to a leaf before being stretched over a vial. In cases where 'treated/washed' squares were used, deionized water was run over the treated surface of parafilm squares before stretching over a vial.

At the onset of the scotophase, pairs of parafilm vials were presented as oviposition sites to newly emerged male/female moth pairs in small cages. Choice pairs were: treated (*B. napus* cv. Westar) vs. untreated, treated/washed vs. untreated, and glossy-treated (*B. napus*, glossy) vs. waxy-treated (*B. napus*, waxy). Moths were allowed to oviposit throughout the 8 hour scotophase. Number of eggs per substrate were compared with paired t-tests.

4.3 Results

Differences in leaf color were evident between glossy and waxy phenotypes (Fig. 4-1); EPTC-treated and control plants had leaves of similar color although leaves of EPTC-treated plants appeared shinier. Epicuticular wax differed in quantity and

morphology between EPTC-treated and control plants (Plate 4-I) and between glossy and waxy phenotypes (Plate 4-II.1, 2, 3, and 4). Although glossy leaves derived either by herbicide treatment or genetic mutation had reduced overall quantities of wax crystals on adaxial and abaxial surfaces, EPTC-treated leaves had fewer rod-shaped and plate-like crystals, whereas glossy genotypes appeared to lack only plate-like crystals. *Sinapis alba* possessed only nondescript beads of epicuticular wax (Plate 4-II.5 and 6).

In laboratory bioassays, moths deposited significantly more eggs on plants treated with EPTC than on control plants (paired t-test, $\alpha = 0.05$, $P = 0.004$) (Fig. 4-2). In field studies of phenotype assays, more eggs were deposited on glossy plants than on waxy. Nested ANOVAs (plant types within cages within plant stages) indicated that plant type explains most of the variation for both years (Table 4-I). No differences were found in transformed data among plant stages for each year but a significantly higher proportion of eggs were deposited on glossy than on waxy phenotypes (paired t-test, $\alpha = 0.05$, $P = 0.001$).

Glossy and waxy plants developed at similar rates, but waxy plants had larger stem diameters (measured at the base) and were taller than glossy plants. Leaf sizes were alike. Significantly more eggs were deposited on the adaxial surface of glossy leaves than on any other plant part regardless of year; numbers of eggs were similar on the abaxial surface of glossy leaves and the adaxial surface of waxy leaves (Fig. 4-3).

Parafilm assays resulted in a significantly higher number of eggs deposited on treated than on untreated parafilm (paired t-test, $\alpha = 0.05$, $P = 0.001$) and also on treated/washed than on untreated parafilm ($P = 0.007$) (Table 4-II). Numbers of eggs

deposited on parafilm pressed to glossy were not significantly different from numbers deposited on parafilm pressed to waxy (Table 4-II).

4.4 Discussion

Chemical composition of epicuticular lipids of the four plant types used in the present study were not determined because it was deemed more important to examine differences in insect behavior to determine the nature of factors influencing moth choices (*e.g.* whether the stimulus is gustatory and polar as previously thought). The three approaches used in this study were devised to isolate epicuticular waxiness from other variables so that the importance of this character could be evaluated for its impact on oviposition by *P. xylostella* (Fig. 4-4). Herbicide-induced wax reduction of *B. napus* changes quantity, not quality, of epicuticular waxes, but cuticular waxes and cutin should not be altered (Flore and Bukovac, 1976, and 1978, respectively). Phenotypically glossy and waxy progeny from a single parental strain of *B. napus* were assumed to have identical chemical make up (including glucosinolate content) except with respect to epicuticular components. There are obvious differences in plant vigor between glossy and waxy phenotypes and, even though waxy plants appeared more robust, they accumulated less than half of all eggs. Parafilm transfers lift only leaf surface components and exclude other factors associated with whole plants such as leaf color. Although parafilm transfers only include those components that will adhere to parafilm, these adherents are apparently sufficient (in all treatment types) for oviposition by *P. xylostella*.

There are a number of ways in which leaf surface waxes could influence *P. xylostella* oviposition: (1) reflectance of light spectra, (2) stability of the insect during oviposition, (3) ease of egg attachment to substrate, (4) availability of polar compounds that are stimulatory to gustatory receptors (*e.g.* glucosinolates), (5) specific chemistry of wax composition (*i.e.* apolar compound) is stimulatory to gustatory receptors and/or to olfactory receptors.

1. Spectral reflectance:

Prokopy *et al.* (1983) found that *D. radicum* landed more frequently on green-leafed plants with spectral reflectance at 500-600 nm, and attributed this largely to spectral sensitivities and/or preferences of *D. radicum*. Waxy varieties that appear bluish-white do not have the same reflectance patterns as greener, glossy varieties. Unlike *Delia* spp., *P. xylostella* oviposition begins at dusk and continues throughout the scotophase in the absence of light (Harcourt, 1957; Pivnick *et al.*, 1990); therefore, spectral reflectance is unlikely to be a contributing factor in oviposition site selection. However, because some eggs could be deposited during the photophase, visual cues are considered here.

Scanning electron micrographs of leaves show waxes differ in structure and amount between glossy and waxy strains. The dark green appearance of glossy plants can be partly attributed to a decrease in wax and partly to an increase in cuticle that compensates for reduced wax to decrease water losses, as in succulent plants (Juniper and Jeffree, 1983). (Herbicide-treated plants did not possess the thicker cuticle that phenotypically glossy plants did which likely contributed to poor plant vigor and premature senescence of leaves.) Although phenotypically glossy plants are a much darker green than their

waxy counterparts, EPTC-treated plants were virtually the same color as control plants. These differences in visual cues do not correlate with host preference and it is likely that vision is not a part of host distinction.

2. Insect stability:

Because an ovipositing female lifts her body during egg deposition (*i.e.* 'ovipositor sweep' and 'oviposition' behaviors, Chapter 3), stability on a substrate is important, particularly on abaxial surfaces where a reliable grasp is necessary for adherance. Insect stability is difficult to quantify even if one compares 'fit' of wax crystals with tarsal dimensions because stability of wax crystals is inconstant and immeasurable. However, *P. xylostella* do not appear to have difficulty with stability on any substrate (personal observation).

3. Egg attachment:

Uematsu and Sakanoshita (1989) found that eggs on glossy-like surfaces (glass, polyvinyl, radish leaf, and cabbage leaf washed with detergent), were flatter (*i.e.* greater area of attachment) than those on waxier surfaces (cabbage, broccoli, and rape leaves). However, they did not consider the degree of waxiness nor other possible influencing variables such as leaf damage from detergent washes, and suggested that waxy substrates are prohibitive to egg deposition because surfaces with less wax allow for better adherance of eggs. Those authors viewed flatness as a cause of egg deposition on leaves with reduced wax, rather than a result of same, which is more likely. Further, flatness may impede development and reduce survivability on glossy surfaces as reported by Eigenbrode *et al.* (1991b). That naturally glossy species such as *S. alba* are not preferred oviposition sites, and that preferred host plants such as *B. oleracea* can have great

amounts of wax, suggest that oviposition site preference is not related to factors influencing egg attachment, or that factor(s) influencing oviposition site preference acts prior to egg attachment.

4. Availability of polar compounds:

Reduced epicuticular lipids may allow for increased availability and/or detectability of polar oviposition stimulants (Eigenbrode and Espelie, 1995), such as glucosinolates, which are not necessarily associated with waxes (van Loon *et al.*, 1992). In herbicide-treatment and phenotype assays, leaves with reduced surface waxes had more eggs, although in both cases plants should have differed only in cuticular makeup. If the quantity of polar stimulants penetrating through to the leaf surface is greater in plants with less wax, one could predict that parafilm transfers of glossy leaves (regardless of whether this is herbicide-induced or genetic) would also have more eggs in choice tests; however, my data show no distinction between parafilm transfers of glossy and waxy leaves.

Polar compounds do not necessarily bind tightly to apolar paraffins and should be easily removed from paraffin surfaces. Compounds that stimulate oviposition by *P. rapae* could not be washed off *B. oleracea* leaves with cold water, but could be removed with boiling water or methanol Renwick *et al.* (1992), and compounds that stimulate oviposition by *D. floralis* could be removed from leaves with hot water (Städler and Roessingh, 1990). Parafilm transfers were very effective in lifting compounds that stimulate oviposition by *P. xylostella* from the surfaces of *Brassica* spp. leaves without apparent leaf disruption, and these stimuli adhere with some affinity because they are not washed from parafilm with cold or warm water. That *P. xylostella* oviposited equally on

parafilm transfers of waxy and glossy leaves suggests that similar stimuli, or at least similarly adequate stimuli, occur on and are lifted from the phylloplane of leaves with either wax type.

Glucosinolates on the phylloplane of *B. oleracea* are responsible for oviposition by *Pieris* spp. (Renwick *et al.*, 1992; van Loon *et al.*, 1992), but polar compounds such as sugars and amino acids that are known to stimulate gustatory receptors of insects, also occur on leaf surfaces (Juniper and Jeffree, 1983). Städler and Roessingh (1990) suggest that phylloplane-dwelling microorganisms may also play a role in host identification by insects. Any or all of these could occur on parafilm transfers, and are likely to occur on leaves regardless of wax content since their existence is independent of epicuticular make-up. Therefore, ubiquitous polar compounds, though they may be stimulatory to ovipositing insects, are likely not the basis for discrimination between glossy and waxy substrates.

5. Availability of apolar compounds:

It is possible that some component of epicuticular wax plays a role in oviposition site selection, especially with respect to the preference for glossy varieties over waxy. Spencer (1996) found that paraffins and/or *n*-alkanes act synergistically with allyl glucosinolate (sinigrin) to increase the number of eggs laid by *P. xylostella* on artificial substrates. Although this seems contrary to all other literature indicating that *P. xylostella* prefer surfaces with reduced wax, it is important to remember that even glossy plants have some waxes (Baker, 1992). Further, apolar compounds can act in olfaction (*e.g.* isothiocyanates) and gustation (*e.g.* surface waxes), but a single compound may not necessarily act in both modalities.

Oviposition by *P. xylostella* is probably not an 'all or none response' triggered by one compound, but rather a graded response to a suite of stimuli (as suggested in Chapter 3) and the host with the greatest number of attractive stimuli receives the most eggs. It may be possible to weight the relative importance of individual stimuli either by further investigation of stimulus complexes similar to this study or by teasing apart individual components and testing each (*e.g.* fractionation and subsequent assays of leaf homogenates).

Eigenbrode *et al.* (1991a) compared leaf extracts of glossy and waxy varieties of *B. oleracea* and found marked differences in composition including the occurrence of two triterpenols, α- and ß-amyrin, only in extracts from glossy leaves. This coincides with differences in larval feeding behaviors, but these triterpenols may also contribute to differences in oviposition behaviors. Interestingly, α- and ß-amyrin occur in glossy but not in waxy *B. oleracea*, but in both glossy and waxy *B. napus* (Holloway *et al.*, 1977). Although not commercially available for bioassay, α- and ß-amyrin are potential candidates for inquiries of gustatory and olfactory cues within epicuticular waxes.

The striking propensity for oviposition on adaxial leaf surfaces is contrary to observations of Harcourt (1957). That abaxial glossy leaf surfaces accumulated egg numbers similar to adaxial surfaces of waxy leaves may provide clues to the nature of surface cues that are important for oviposition site preference.

Table 4-I. Nested ANOVAs: field trials of ovipositing *P. xylostella* given choices of glossy and waxy phenotypes (types), replicated 'n' times (cages) for rosette, early flowering, and seed pod development (stages); upper table is 1995, lower is 1996. (See Appendix IV for calculations.)

Sources of variation	*df*	*SS*	*MS*	*F ratio*	Significance
Among groups (stages)	2	294.45	147.23	1.69	ns
Among subgrps (cages)	9	780.18	86.69	0.54	ns
Among subsubgrps (types)	12	1949.7	162.48	5.83	P < 0.0005*
Among subsubsubgrps (samples)	96	2673.6	27.85		
Total	119	5697.93			

Sources of variation	*df*	*SS*	*MS*	*F ratio*	Significance
Among groups (stages)	1	136.89	136.89	0.77	ns
Among subgrps (cages)	8	1414.6	176.83	0.22	ns
Among subsubgrps (types)	10	7966.3	796.63	9.26	P < 0.0005*
Among subsubsubgrps (samples)	80	6880.0	86.0		
Total	99	16397.8			

* from Zar, 1984.

Table 4-II. Number of eggs deposited per female per night on parafilm substrates (n = 12 for each pair of substrates).

Choice assay:			Mean (± standard error) number of eggs :		
Substrate 1	vs.	Substrate 2	Substrate 1	Substrate 2	Significance
untreated	vs.	treated	5.6 ± 4.3	25.1 ± 6.3	0.001
untreated	vs.	treated/washed	1.0 ± 0.6	19.9 ± 5.7	0.007
glossy treated	vs.	waxy treated	8.6 ± 2.1	7.4 ± 1.5	ns

paired t-test; $\alpha = 0.05$.

Figure 4-1. Alternating rows of waxy (centre row) and glossy (left and right) phenotypes of *B. napus* in a field plot at Vegreville, Alberta.

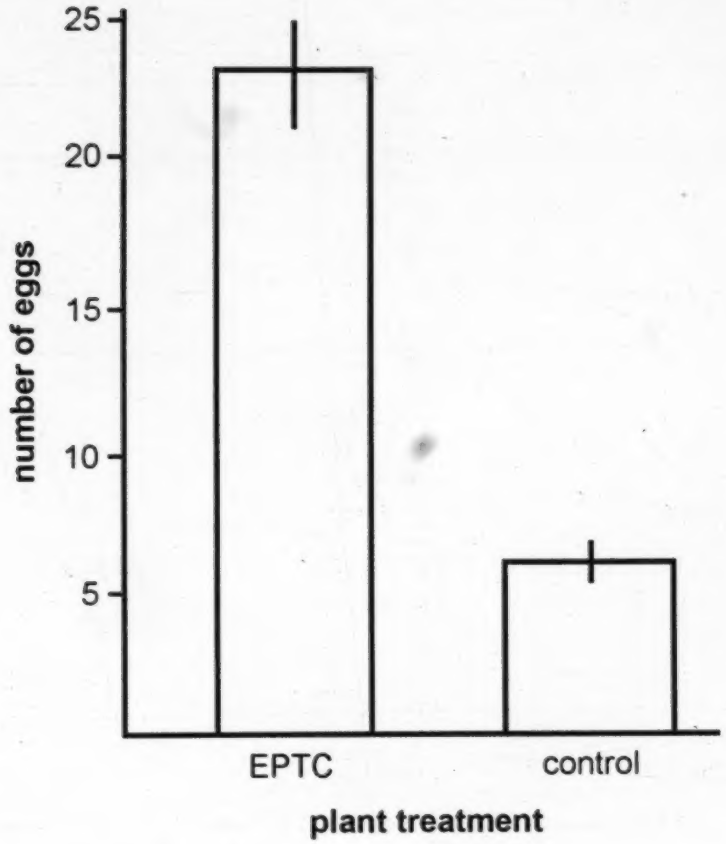


Figure 4-2. Number of eggs (± standard error) deposited on control and EPTC-treated plants in choice assays (n = 7 replicates).

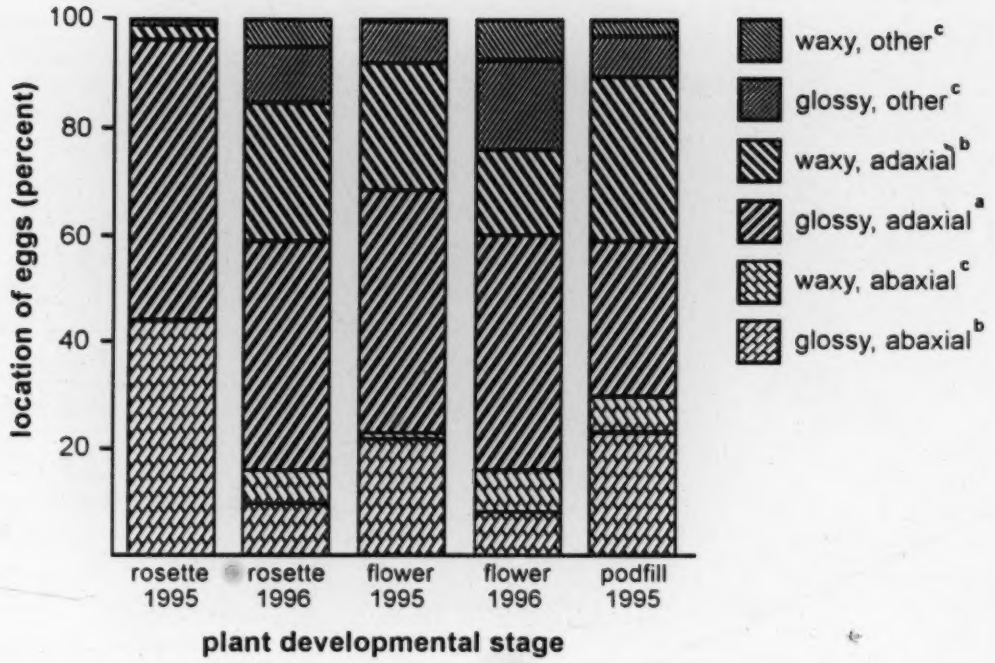


Figure 4-3. Distribution of eggs on plants at three growth stages: late rosette (growth stage 3.1 of Harper and Berkenkamp, 1975), early flowering (growth stage 4.1), and early seed pod development (growth stage 4.3) on five randomly chosen waxy plants and five randomly chosen glossy plants per cage. (Note: trials with early pod development were not conducted in 1996.) Superscript letters denote significance; Kruskal-Wallis, $\alpha = 0.05$, $P < 0.0001$.

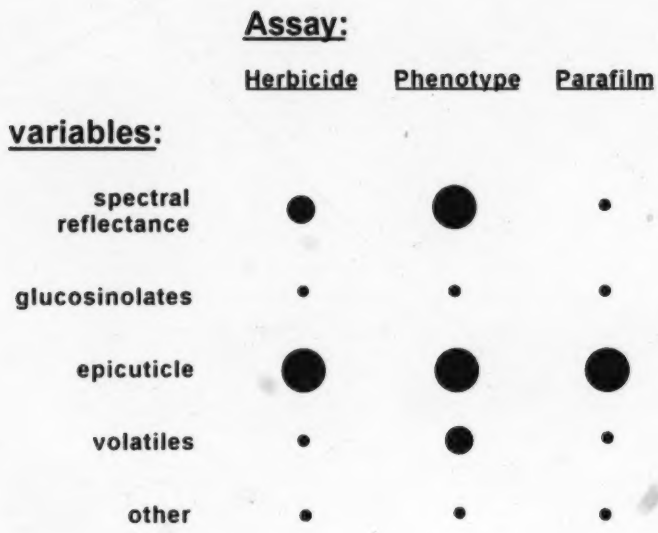


Figure 4-4. Relevant variables addressed by each assay type; assay pairs are control vs. EPTC-treated plants (herbicide), glossy vs. waxy *B. napus* (phenotype), and glossy vs. waxy *B. napus* parafilm presses (parafilm). Dots represent variables considered in each pair-wise assay; size of dots represents degree of contrast between assay pairs.

Plate 4-I. Scanning electron micrographs of adaxial leaf surfaces of control and EPTC-treated *B. napus* cv. Westar: 1) control, bar = 20 μm; 2) control, bar = 5 μm; 3) EPTC-treated, bar = 20 μm; 4) EPTC-treated, bar = 5 μm.

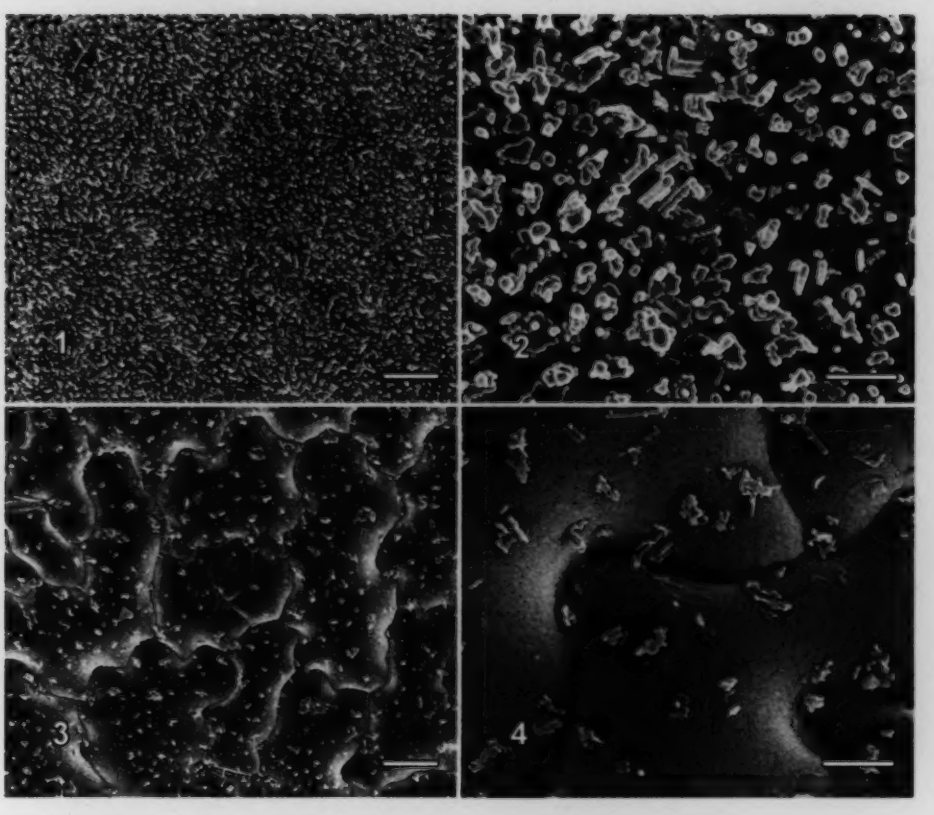
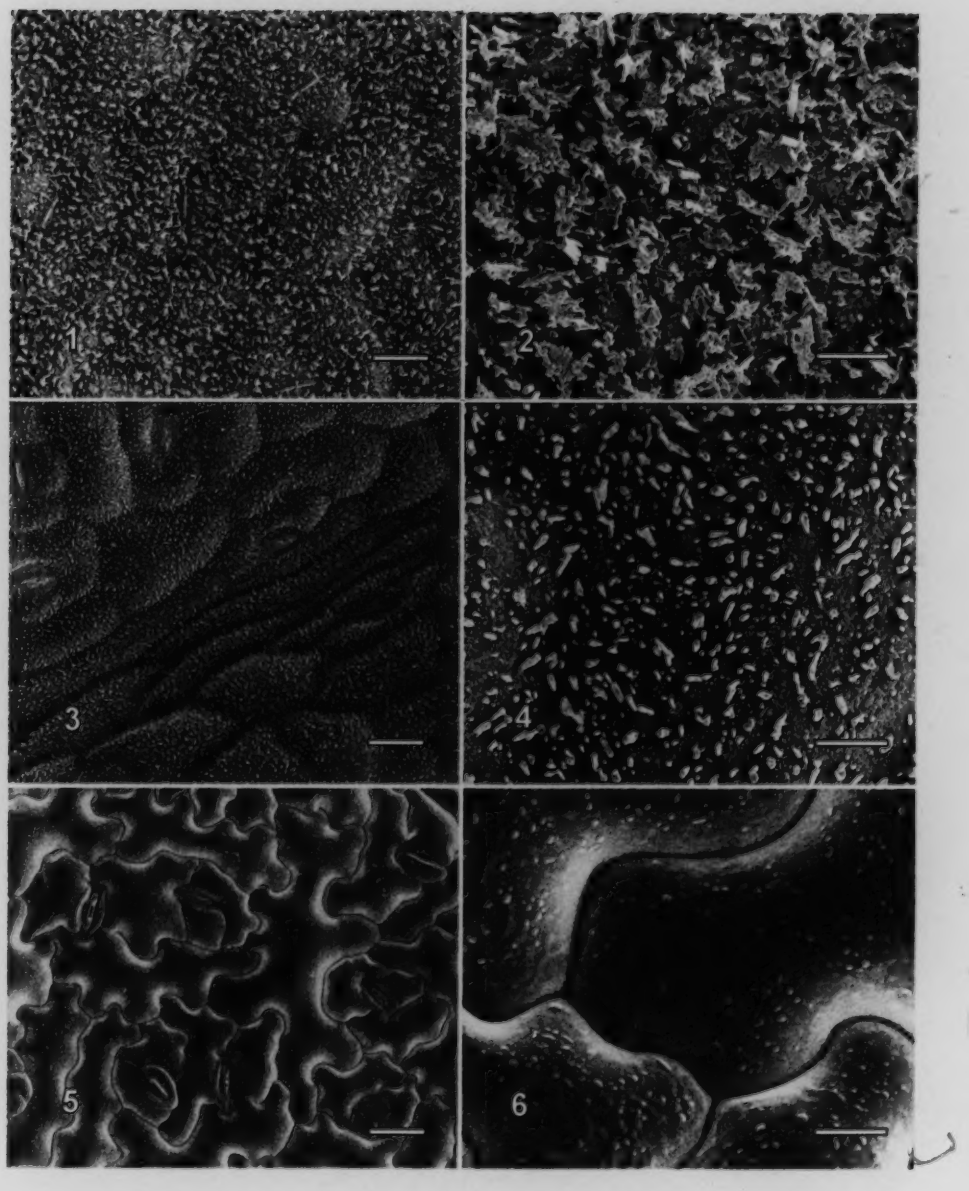

1
2
3
4

Plate 4-II. Scanning electron micrographs of adaxial leaf surfaces of: 1 and 2) waxy *B. napus*, bars = 20 μm and 5 μm, respectively; 3 and 4) glossy *B. napus*, bars = 20 μm and 5 μm, respectively; 5 and 6) *S. alba* cv. Ochre, bars = 20 μm and 5 μm, respectively.


1
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Chapter 5:

Antennal sensilla and electroantennogram responses of *Plutella xylostella* to headspace extracts of host and non-host plants

5.1 Introduction

It has long been accepted that isothiocyanates are olfactory cues for host location by cruciferous insects (*e.g. C. assimilis*, Bartlett *et al.*, 1993; *D. brassicae*, Wallbank and Wheatley, 1979; *D. brassicae*, Finch, 1978; *B. brassicae*, Pettersson, 1973; *P. cruciferae*, Feeny *et al.*, 1970; and *P. xylostella*, Gupta and Thorsteinson, 1960). However, intact *B. napus* plants produce only trace amounts of isothiocyanates (Tollsten and Bergström, 1988) and most cruciferous insects readily oviposit and feed on this and other plants (such as cultivars of *B. rapa*) that produce few or no isothiocyanates.

The plant chemosphere (*i.e.* headspace) of *Brassica* spp. is a complex blend of more than twenty volatiles (Tollsten and Bergström, 1988) and it is this complexity that makes identification of physiologically- and behaviorally-active compounds problematic. Gas chromatography (GC) has been used to separate volatiles for use in behavioral and electrophysiological studies. In early studies, GC effluents were sequentially trapped and these partial separations used in bioassays *vis-a-vis* elimination of non-active fractions. Sequentially trapped GC effluents from headspace volatiles of intact and homogenized *B. juncea* allowed partial identification of compounds that may be stimulatory to *P. xylostella* (Pivnick *et al.*, 1994). However, this technique has some disadvantages such as a decrease in resolution and the need for supplementary separations to identify active

components, especially if more than one exists in a single fraction. Arn *et al.* (1975) found that capillary GC produces sufficiently short rise times and separation of components to evoke good electroantennographic (EAG) responses with simultaneous recording (*i.e.* GC-EAG). This coupled technique provides precise information of electrophysiologically active components in a given chromatogram, and eliminates mechano- and thermosensory interference.

Ovipositing *P. xylostella* perform intricate antennal movements (Chapter 3) that may be involved in detecting high molecular weight, low volatility compounds such as those indicated by Pivnick *et al.* (1994).

The objectives of this study were: to describe type and locations of olfactory sensilla of *P. xylostella,* to investigate what chemosphere components of host and non-host plants are detected by the antennae, and to correlate antennal perceptions with oviposition activity in the presence of these chemosphere extracts.

5.2 Materials and Methods

5.2.1 Insects

Insects used for antennal sensillar descriptions were reared as described in Chapter 2.

Insects used for electrophysiology were reared in cages in glasshouses with sodium vapour lamps to achieve day lengths greater than 12 hours. Potted *Brassica* spp. and honey-water were provided as in previous studies. Pupae were collected and allowed to eclose in the absence of *Brassica* odors in cylindrical containers (14.5 cm x 14.5 cm

diam) of plexiglass and nylon mesh. Newly emerged adults were sexed and placed singly in glass vials. Animals were chilled at 4 °C immediately prior to use.

Animals used for bioassays were reared as in Chapter 2 and allowed to mate under conditions similar to those described in Chapter 3.

5.2.2 Scanning Electron Microscopy

Critical point dried antennal specimens (see Appendix III-1) were mounted on aluminum stubs, double sputter-coated with gold (100 Å total) and viewed with either a Cambridge S-250 Stereoscan or a JSM-6301FXV field emission scanning electron microscope with an accelerating voltage of 5 kV.

5.2.3 Volatile collection

Alternate rows of glossy and waxy *B. napus* seed (S2 seed of Chapter 4) were hand seeded in field plots at Wädenswil, Switzerland. Leaves were harvested for volatile collection at early flowering (growth stage 4.1, Harper and Berkenkamp, 1975). Potted plants from the same seed were started every week in glasshouses to ensure a continuous supply of foliage from rosette to early flowering plants (growth stages 3.1 - 4.1, Harper and Berkenkamp, 1975).

A push-pull system, similar to that described by Turlings *et al.* (1990), of compressed air (Carbagas) and a vacuum pump (Model 400-1902; Banant Co., USA) was used to collect plant volatiles. Compressed air was purified through an activated charcoal gas purifier (120 cc; Alltech Associates Inc), humidified through a 250 ml gaswash flask, and split to pass through one of two four-litre glass containers that had approximately 40 g fresh, chopped plant material (from either glasshouse or field plants) in nylon netting (1 mm mesh) suspended at approximately 1/3 the height of the container. A gaswash flask

was attached to one outlet of each container to monitor pressure; airflow settings were regulated for slightly positive pressure to prevent backflow. Adsorbent traps were made with 50 mg of Super Q 80/100 (Alltech Associates Inc.) fixed between two fine-mesh stainless steel screens in 10 cm of glass tubing (3 mm inner diameter). Traps, inserted into silicon-seal trap holders, were fixed to the outlet of the plant containers. Post-trap airflow from the two plant containers converged just prior to arrival at the flow meter. An empty basin, similar to that containing plant material, was set between the flow meter and the vacuum pump as a ballast tank to buffer small isochronal fluctuations in flow from the vacuum (Fig. 5-1). All tubing and connectors were either glass or teflon, and were periodically cleaned and checked for contamination (Appendix V-1).

The collection system was set in a laboratory growth chamber with sodium vapor lamps (22°C, and 70 % R.H). Flow rate during collections was maintained at 0.5 - 0.6 l/min for two hours (see Appendices V-2 and V-3 for reference checks). Collections took place in the mornings when soil moisture of potted plants was moderate to dry.

Immediately after collection, traps were eluted with 150 µl of dichloromethane (Merck), reduced to 20 µl with N_2 (Carbagas), diluted in 100 µl of hexane (Merck), and stored in reactivials™ at -12 °C until use (see Appendix V-7).

A similar system was set up for collections from intact plants but, because these were not used in GC-EAG experiments, is not reported here.

5.2.4 Gas Chromatography (GC)

A gas chromatograph (Carlo Erba: Fractovap Series 2150, Electrometer Model 180, Lt. Programmer Model 232) was equipped with a flame ionization detector (FID), a 10 m retention gap (BGB Analtyk AG, Switzerland), and a 30 m x 0.32 mm id DB-1 capillary column (J &W Scientific Inc., USA). Column eluent was split with a glass universal pressfit splitter (Restek) to effect a split ratio of 2 EAG:1 FID. Carrier gas was helium and make up gas hydrogen with a linear velocity of 0.5 kg/cm^2. A modified port functioned as a second outlet through which the capillary column passed to feed (2 mm) into a glass odor delivery tube (4.8 mm diameter) which directed the airstream to an animal preparation (Fig. 5-2). Initially, the glass odor delivery tube was designed so that the distance between the entrance hole of the capillary column and the opening to the animal preparation was 30 cm. However, signal-to-noise ratios and EAG responses with this setup were poor and modifications were necessary to shorten the glass odor delivery tube as follows. The EAG arm of the capillary column exited the GC oven through the second GC outlet and passed through a length of stainless steel tubing (~ 1.5 mm inner diameter). Further modifications were made to the outlet to stabilize the capillary column and prevent its breakage (*e.g.* a steel strip was coiled around the base of the stainless steel tubing and set into the outlet fixture to prevent column vibrations). The stainless steel tubing was encased in copper tubing and bent at four 135° angles to form a bridge-shape. The entire structure was wrapped with a fibreglas heating tape that was connected to a temperature control transformer (Model TCI-88/3R, Systag, Switzerland), insulated with fibreglas and teflon, shielded with aluminum foil and grounded. Temperature was monitored with a thermocouple (Pt 100 for 300 °C maximum; Systag, Switzerland) set in

the copper tubing. This setup effectively allowed the capillary column to be heated at increments paralleling that of the GC oven, and extended the working distance between the GC outlet and animal preparation to shorten the carrying distance of the glass odor delivery tube. The shortened delivery tube had a hole in its sidewall 5 cm from the opening to the antennal preparation (rather than the initial 30 cm) to accomodate entrance of the capillary (Fig. 5-2, rectangular inset).

The temperature program for standards was 50°C for 2 min, 10°C/min to 80°C, 15°C/min to 110°C for 5 min and the temperature program for plant extracts was 50°C for 2 min, 10°C/min to 80°C, 15°C/min to 230°C for 5 min. These programs provided good separation and rise times, and allowed sequential runs of standards and extracts without compromising the longevity of animal preparations (Appendix V-6). The column was baked at 260°C for 20 minutes between animal preparations. Both outlets were held constant at 275°C during operation.

The GC signal was amplified (10x) and filtered (low pass with a cornering frequency of 0.1 Hz) to a two-pen chart (Philips PM8262 Xt) and computer recorders (SuperScope II software, GW Instruments) (Fig. 5-3).

5.2.5 Electroantennography (EAG)

Compressed air (Carbagas) was humidified through a gaswash flask. Airflow was regulated at 900 ml/minute to produce a continuous airstream over the antennal preparation (824 mm/sec). Upon exiting the gaswash flask, the airstream was split into continuous and complementary airstreams at a 9:1 (continuous:complementary) ratio which converged prior to occurrence at the odor delivery tube. The complementary airstream could be momentarily diverted via a magnetic switch controlled with a foot

pedal, so that instead of its usual convergence with the continuous airstream, the complementary airstream entered the glass odor delivery tube via a Pasteur pipette inserted into a small hole in the delivery tube (see Guerin and Visser,1980). The Pasteur pipette contained a prepared stimulus on filter paper which was swept into the odor delivery tube when the foot pedal was depressed (Fig. 5-2). Puffs of stimuli delivered to animal preparations in this manner served as external system checks only.

Electrodes were made from thin-walled, filamented borosilicate capillaries (Clark Electromedical Instruments) drawn with a vertical pipette puller (Model 700C; David Kopf Instruments). Freshly drawn capillaries were filled with physiological saline (Appendix I-5) and fitted over Ag-AgCl wire in electrode holders.

Animals were mounted in a groove on a plexiglass block and secured with small strips of adhesive tape. Wings and forelegs were removed immediately prior to mounting. With the aid of micromanipulators and a stereo microscope, the recording electrode was inserted into the pedicel-flagellar intersegmental membrane of the antenna (Fig. 5-2, circular inset) and the indifferent electrode inserted into the distal end of the antenna after removal of the first 1-4 segments. (This electrode position is opposite to conventional setups and was countered by switching polarity at the chart recorder.) Hemolymph congealed and sealed the electrode to the antenna within 60 seconds to give a reasonable signal-to-noise ratio, and allowed the antenna to be manouvered into the humidified airstream via micromanipulators.

The recording electrode was attached to a preamplifier (10x, powered by six three-volt DC batteries) and the indifferent electrode was attached to a pulse generator which was also attached to the preamplifier. (The pulse generator served as an external

system check.) The EAG signal was amplified and filtered (high pass with cornering frequency of 0.02 Hz) and sent to an oscilloscope and two-pen chart recorder. Unfiltered signal was sent to the oscilloscope and computer recorder (Fig. 5-3).

5.2.6 GC-EAG

A 0.1 mM mixture of three isothiocyanates (allyl, butyl, and phenyl) in hexane was used as a standard (Appendix I-6), and this was injected immediately preceding and succeeding every plant extract injection. The use of standards permitted verification of a working preparation and calculation of time lags between FID and EAG traces for subsequent alignments. Trials with a second standard (Appendix I-7), a mixture of five terpenes (0.1 mM concentrations of (-)-borneol, (+)-terpinen-4-ol, α-terpineol, 4-allylanisole, and verbenone), were performed with some animals before or after runs with isothiocyanate standard and plant extracts.

Because retention times were slightly variable among chromatograms, EAG response patterns were standardized (after Baur *et al.*, 1993) as follows. One FID trace was marked for every location in which the corresponding EAG trace showed an EAG response. These locations were assigned letter labels (*e.g.* A, B, C) and each EAG trace was assigned parallel labels (Fig. 5-4). EAG peaks were only considered to be responses to the eluting compounds if they were at least two times the baseline noise immediately before and after. Amplitudes of EAG responses to extract eluents were measured and expressed as a proportion of the corresponding EAG response to butyl isothiocyanate (*i.e.* $EAG_{\text{extract eluent}}$ mV / $EAG_{\text{butyl ITC}}$ mV). Data were arcsine-transformed (Zar, 1984) and responses compared with a two-way ANOVA.

Absolute quantities of eluents were not determined due to overlap between some compounds and overloading of some FID peaks (making peak shape impossible to determine).

5.2.7 Gas Chromatography - Mass Spectrometry

Gas chromatography - mass spectrometry (GC-MS) for peak identifications was performed using a VG Tribid mass spectrometer (on-column injection) equipped with a 30 m x 0.25 mm id DB-1 capillary column. Carrier gas was helium and EI spectra were recorded at 70 eV. Extracts used for identification were mixtures of several glossy *B. napus* collections, one of which combined collections from field- and glasshouse-grown plants. Temperature program was 50 °C for 2 min; 10 °C/min to 80 °C; 5 °C/min to 260 °C.

5.2.8 Bioassays

Oviposition arenas contained substrates that were treated with 10 mM sinigrin (see Chapter 3) and a plant extract as follows. A small drop of paraffin oil was placed adjacent to the sinigrin-treated site and allowed to reach maximum attenuation at which time 3 µl of one of the plant extracts (glossy *B. napus*, waxy *B. napus*, *S. alba*, or *D. carota*) was added to the oil stain. Hexane was allowed to evaporate from the substrate (approximately 60 seconds; 45 seconds resulted in 50% mortality) before it was placed in the oviposition arena containing a mated 3-4 day old female moth. Number of eggs deposited during the first hour of and entire (eight-hour) scotophase were recorded and compared with one-way ANOVAs.

5.3 Results

5.3.1 Antennal description

Male and female antennae have 35 - 38 flagellar segments with no obvious differences in appearance between the sexes. Every flagellar segment is circumscaped at the apical end by a band of seven to ten stacked scales. The innermost, or bottom, layer consisted of short scales with the longest scales in the outermost, or top, layer so that *in situ* the outer scales shield all others from view and give the illusion of a single band of scales projecting anteriorly toward the next segment, entirely covering the intersegmental joint. The dorsal surface has a second, incomplete band of scales that does not extend to the ventral surface. This band is comprised of four to six layers of scales with only the outermost layer apparent, like the complete band (Plate 5-I.1 and 2). Sensilla are dispersed throughout the antennae between scales, and therefore, are more abundant on the ventral face where scales are lacking (Plate 5I.3 and 4). Seven sensillar types were seen on the antennae of both male and female *P. xylostella*: chaetica, styloconica (two types), coeloconica, trichodea (two types), and a previously undescribed type.

The distal segment of the antennae has a small protuberance that appears to be only cuticular sculpting and spines when viewed from any aspect other than perpendicular (Plate 5-I.5). The apex bears three socketed aporous styloconic sensilla (< 1.0 μm diameter) set equidistant from each other (Plate 5-I.6 and 7).

Chaetic sensilla (ca. 27 μm in length) have a slight S-shaped curvature (Plate 5-II.1) and are socketed (Plate 5-II.2). The sidewall is terraced nearly entirely from base to tip. A single terminal pore of ca. 120 μm is associated with five fingers which are always adducted in SEMs (Plate 5-II.3). There are four chaetic sensilla on every

segment, arranged somewhat in a horizontal row on the ventral face, except at the most distal segment which has up to 14 surrounding the apex (Plate 5-I.5).

All flagellar segments, except the last, bears one styloconic sensillum set in a large peg-like base of 6 μm x 12 μm (Plate 5II.4). These are situated rostro-ventrally (at the outer limits of the partial band of scales) at the apical edge of flagellar segments and bend across intersegmental joints to the next segment (Plate 5-I.3 and 5). Pores were not evident.

Small (ca. 1 μm diameter) bud-shaped formations set in sockets (similar to styloconic sensilla of the apex) are present at the apical extremity of some segments, but are rare in occurrence (Plate 5-II.5). Pores were not evident.

Coeloconic sensilla consist of a short peg with a scalloped sidewall that is set in a small depression (ca. 7.5 μm diameter) and surrounded by a number of cuticular spines (Plate 5-II.6). Pores were not obvious but may exist between scalloped ridges of its sidewall. Distribution of these sensilla appeared to be random but each flagellar segment had at least two and as many as eight.

Trichodeal sensilla are present in two forms (Plate 5-III). Type I is longer (ca. 31 μm) than type II (ca. 18 μm), has a larger base (type I ≈ 2.0 μm, type II ≈ 1.2 μm), and tapers to a smaller diameter tip (type I = 0.4 μm; type II = 0.75 μm). Type I has annular ridges and small pores (ca. 25 nm) distributed throughout the length but only on opposing sides of the sensillum (Plate 5-III.1, 3, and 5) whereas the pores of Type II occurs only in longitudinal grooves (Plate 5-III.2, 4, and 6). It is also likely that type II hairs are thin-walled because these sensilla were often collapsed (Plate 5-II.1).

5.3.2 GC

All headspace extracts had several major peaks and many minor peaks. Chromatograms of glossy and waxy *B. napus* collections did not appear to differ qualitatively but did differ quantitatively. Collections from leaves of field-grown plants produced substantially larger peaks than those of glasshouse-grown plants, and collections from glossy leaves had larger peaks than those of waxy plants (*i.e.* field glossy > field waxy > glasshouse glossy > glasshouse waxy) (Fig. 5-5). Chromatograms of *S. alba* were quite similar to *B. napus*, but had a lesser number of, particularly major, peaks (Fig. 5-6, b). Chromatograms of *D. carota* extracts (Fig. 5-6, c) differ completely from *B. napus* and *S. alba.*

5.3.3 GC-EAG

EAG responses to isothiocyanate standards were not equal. Butyl isothiocyanate elicited the greatest EAG responses (independent samples t-test, $\alpha = 0.05$, $P = 0.043$) although responses from males were only approximately half that from females. Responses to allyl isothiocyanate were smaller from both sexes but, because responses by males were not reliably produced, only that of females was compared to butyl isothiocyanate (paired samples t-test, $\alpha = 0.05$, $P < 0.0001$). There was no response to phenyl isothiocyanate in either sex (Table 5-I), nor were there responses to any of the terpene standards (data not shown).

Antennae responded to at least ten compounds in extracts of *B. napus* and five of these were relatively minor components (Fig. 5-4). *Brassica napus* compounds eluting at A/B, D, G, H, I, and K consistently produced EAG responses in > 70% of moths tested, and compounds C, E, and F elicited responses in > 30% of moths tested. (Eluents A and

B were indistinguishable because they co-occurred within a large broad peak and therefore, have been combined for analyses.) Responses to *S. alba* extracts were similar except no EAGs were observed at elution points D and F, and EAGs occured at elution points P, J, and L which were not observed with *B. napus* extracts (Fig. 5-7). *Daucus carota* extracts elicited few EAGs, but those occuring in > 70% of moths had elution times that coincided with those of peaks A/B, F, and K (Fig. 5-7).

Responses to plant extracts were somewhat smaller than responses to the standard (*i.e.* butyl isothiocyanate) and responses to some eluents were quite variable (Fig. 5-8). Eluents A/B consistently elicited the largest responses within *B. napus* tests, and eluent I the largest with all host plant extracts. Eluent H elicited large EAGs with *B. napus* extracts but much smaller EAGs with *S. alba* extracts (ANOVA, $\alpha = 0.05$, $P = 0.096$), and the reverse was true for eluent E (ANOVA, $\alpha = 0.05$, $P = 0.004$) (Fig. 5-7).

5.3.4 GC-MS

A number of compounds in *B. napus* extracts were identified (Table 5-II). Eluting compounds A, B, C, and D are fatty acid derivatives and are in high concentrations in all *B. napus* and *S. alba* extracts. However, some of the very minor components elicited relatively large EAGs and not all of these were successfully identified (Table 5-II).

5.3.5 Bioassays

Moths provided with *D. carota* headspace odors did not oviposit. Numbers of eggs deposited throughout the eight hour scotophase were not significantly different among extracts and paraffin oil controls (Table 5-III). However, a significantly greater number of eggs were deposited in the first hour by moths presented with either of the *B.*

napus extracts than those presented with *S. alba* or paraffin oil only (ANOVA, $\alpha = 0.05$, $P = 0.002$) (Table 5-III).

5.4 Discussion

Schneider and Steinbrecht (1968) proposed a four-step approach for determining olfactory localization in insects:

1) identification of sensilla,

2) recording summated electrical responses,

3) identification of cellular function within a sensillum, and

4) biochemical identification of receptors.

This study investigated the initial two objectives plus a third, behavioral significance, not included in Schneider and Steinbrecht's schematic.

Morphologically defining sensilla is problematic in that morphological type does not affirm function, and external similarities between sensilla often do not reflect internal similarities (Altner and Prillinger, 1980). Despite this, categories of sensilla have been established largely on the basis of external morphology, and functional relationships established secondarily. Four types of olfactory sensilla were categorized by Schneider and Steinbrecht (1968): trichodea, basiconica, coeloconica, and placodea. Styloconic and ampullaceous sensilla have also been classified as olfactory (Lewis, 1970) but styloconic sensilla can be thermo-, hygro-, and/or chemoreceptive (see Altner and Prillinger, 1980). The present study uses classical categorical nomenclature as that of Callahan (1975) to maintain coherence within this body of literature.

Antennal sensilla of *P. xylostella* are strikingly similar to those of *Yponomeuta* spp. (Cuperus, 1986; van der Pers, 1980) and sensilla of *P. xylostella* are similar (in external structure and location) to those of other Lepidoptera (*e.g. Helicoverpa assulta*, Koh *et al.*, 1995; *O. nubilalis*, Hallberg *et al.*, 1994; *Yponomeuta* spp., Cuperus, 1986; female *Mamestra configurata*, Liu and Liu, 1984; *Agrotis segetum*, Hallberg, 1981; *Cydia nigricana*, Wall, 1978; and *C. fumiferana*, Albert and Seabrook, 1973).

Although functions of specific sensillar type were not determined, there is no reason to believe that *P. xylostella* sensilla would differ functionally from those on other lepidopteran antennae. Terminal styloconic sensilla of *H. assulta* were characterized as hygro- and thermoreceptive based on external and internal structures (*i.e.* mitochondria, ciliary basal bodies, and lamellae; Koh *et al.*, 1995), and because of their specific placement and similarity, those of *P. xylostella* probably have similar functions. Aporous sensilla are not generally considered to be chemoreceptive (Zacharuk, 1980), but there are many cases in which multiporous sensilla are olfactory (*e.g. Dacus oleae*, Crnjar *et al.*, 1989; *Yponomeuta* spp., van der Pers, 1980) and it is likely that at least some multiporous sensilla on *P. xylostella* antennae are olfactory. Scanning electron microscopy of other de-scaled body parts of *P. xylostella* revealed a lack of sensilla (except those described in Chapter 6). Behavioral (Chapter 3) and sensillar descriptions of *P. xylostella* antennae suggest that they are active in host plant selection.

Plutella xylostella is apparently attracted to isothiocyanates (Pivnick *et al.*, 1994; Gupta and Thortsteinson, 1960), and Pivnick *et al.* (1994) concluded that *P. xylostella* did not differentiate between traps baited with various isothiocyanates (allyl, 2-phenylethyl, and *n*-propyl) because similar numbers were caught in traps baited with similar

concentrations of any of the isothiocyanates. However, in low-rate release traps, numbers of moths trapped did not differ from control traps that lacked isothiocyanates. In the current study, electroantennogram responses to allyl and butyl isothiocyanates were quite different from each other and no responses occurred with phenyl isothiocyanate indicating that moths have the capacity to differentiate between at least some isothiocyanates. It is unknown how or if the ability to distinguish among isothiocyanates is important in host plant selection.

There are also differences in sensitivity of males and females to at least two isothiocyanates (allyl and butyl isothiocyanate) but this does not imply that isothiocyanates are more attractive to females. Electroantennograms are indicative of the presence and activity of receptors for a given stimulus. However, because a single EAG event is a summation of electrical potentials (Schneider, 1969) rather than a response by a single sensory cell, no information is procured regarding sensillar character, absolute number of cells responding, or sensitivity of those cells. Instead, strength of an EAG (*i.e.* amplitude) provides some information regarding relative sensitivity of cells to a particular compound or relative number of cells responding. For example, consecutive EAG recordings from *P. brassicae* in which the recording electrode was situated at various sites along the antennae yielded progressively smaller EAGs as the length between electrodes decreased (Behan and Schoonhoven, 1978). Similar phenomena were seen in *Trichoplusia ni* (Lepidoptera: Noctuidae) when stimulus application was confined to a number of segments and when segments were progressively removed from antennal preparations (Mayer *et al.*, 1984). Male and female *P. xylostella* antennae are similar in length and smaller EAGs in response to equal concentrations of isothiocyanates must be a

result of either fewer cells responding or smaller electrical potentials per cell. Palaniswamy *et al.* (1986) reported greater responses from female *P. xylostella* to homogenized plant extracts. Sexual dimorphisms in EAG responses are commonly reported in pheromone investigations but there are few such reports with reference to plant volatiles (*e.g. C. assimilis*, Evans and Allen-Williams, 1992).

Often, male moths display morphological modifications for detecting female pheromones, such as specialized terminal organs (*M. configurata*, Liu and Liu, 1984) or large increases in the number of available sensilla and surface area as in *Antherea polyphemus* (Lepidoptera: Saturniidae) (Kaissling, 1987). Chow *et al.* (1984) found a greater number of multiporous sensilla trichodea (type I and type II were not distinguished) on antennae of male *P. xylostella* than on females, and noted that male antennae produced EAGs when stimulated with synthetic pheromone compounds (Z-11-hexadecenyl acetate and Z-11-hexadecenal) but females did not. It is possible that males are able to detect host plant volatiles but that most olfactory cells of male antennae are devoted to procuring mates which would account for smaller EAGs to plant volatiles (*i.e.* fewer 'generalist cells' and a greater number of 'specialist cells' (Schneider and Steinbrecht, 1968)). Trichodeal sensilla are thought to have many generalist cells but there is evidence that these sensilla in males also harbor pheromone-receptive cells (Den Otter and Thomas, 1979; Schneider and Steinbrecht, 1968). Electroantennograph traces from male moths exhibited similar numbers of EAG peaks to *B. napus* extracts as from female moths but because EAGs to standards were smaller, relative sizes of EAG responses to plant compounds were not determined. To do so would produce spurious and uninformative results. However, all plant compounds that elicited EAG responses by

females also elicited responses by males. Sample sizes of male responses to *S. alba* and *D. carota* were too small to analyze (data not shown).

It is no surprise that extracts from *S. alba* and *B. napus* show similarities to each other but not to *D. carota* because *S. alba* and *B. napus* are taxanomically unrelated to *D. carota*.

That glossy *B. napus* leaves apparently emit more volatiles than waxy leaves is probably related, in part, to lower transpiration rates in waxy plants (Jeffree, 1986). Interestingly, field-grown plants produced a greater quantity of volatiles than glasshouse-grown plants but the reason for this is unknown. All collections began at early morning. Glasshouse-grown plants had moderate to dry soil at sampling and, although field conditions could not be controlled, field plants were not sampled if surface soil moisture content was high because slightly moisture-starved plants produce more volatiles (T.C.J. Turlings, personal communication). (That is, if it had rained that morning or the previous evening, plants were not sampled.) Field plants used for collection were slightly developmentally advanced (growth stage 4.1; Harper and Berkenkamp, 1975) than some glasshouse plants (growth stage 3.1 - 4.1), but it is more likely that this would equate to a different suite of compounds and not necessarily to different quantities of compounds (see Tollsten and Bergström, 1988). Wallbank and Wheatley (1976) found that older plants of *B. oleracea* var. *botrytis* produced lower concentrations of volatiles which would suggest that age does not explain the productivity of *B. napus* field plants. Perhaps field-grown plants were healthier, but differences in plant vigor were not obvious.

Some volatile collections from whole plants (data not shown) were performed with glasshouse-grown plants (at growth stages 3.1, Harper and Berkenkamp, 1975). Extracts from these collections provided few peaks, much lesser than extracts from chopped leaves at similar developmental stages. Similar results with whole and chopped cabbage leaves were reported by Finch (1978). Tollsten and Bergström (1988) reported a greater diversity of compounds from whole *Brassica* plants, but fewer terpenoids and more fatty acid derivatives and nitrogenous compounds were emitted from macerated *B. napus* plants. Antennal responses to whole plant extracts were not performed but would be useful in determining what host plant blends are sensed by *P. xylostella* in field situations.

Despite quantitative differences, glasshouse- and field-grown plants elicit similar responses from moth antennae. Responses regularly occurred for eluents A/B, C, D, E, F, G, H, I, and K, although fewer moths responded to C, E, and F from waxy *B. napus* extracts. Eluents E and F occurred in very small concentrations and since waxy plants produced overall smaller quantities of volatiles, eluents E and F may be in concentrations that are too small for detection by *P. xylostella*. This may also be true of eluent C but, because it overlaps with other compounds in the chromatogram, its relative volume is not easily resolved. EAGs produced by eluents M, N, and R were infrequent among moths tested with any *B. napus* extract.

Interestingly, all moths tested produced EAGs at eluents H and I which occurred in minute concentrations (lower than that of E and F) but produced relatively large EAGs suggesting a greater sensitivity to and/or a large number of cells responding to these host

plant components. In fact, these components could be key stimuli for host plant identification by *P. xylostella*.

Sinapis alba extracts elicited EAGs at similar elution points to those of *B. napus* eluents A/B, C, E, G, H, I, and K, but only about half of moths tested showed any response to elution point H with *S. alba* extracts which suggests that the components at elution point H is not the same in *B. napus* and *S. alba* extracts. No EAGs were evident at elution points D or F, and three EAG responses were unique to *S. alba* extracts (P, J, and L). Such disparities may play a role in *P. xylostella*'s preference for *B. napus*. Perceived volatiles are not necessarily attractants but can be repellents (Baur *et al.*, 1993) and eluents P, J, and/or L of *S. alba* extracts could have behaviorally repellent effects.

Daucus carota extracts evoked few EAGs from *P. xylostella*, but those compounds eliciting EAGs had elution times similar to those of *B. napus* eluents A/B, F, and K.

Several components of *B. napus* (glossy) extracts were identified. Eluents A/B, C, D, and F are fatty acid derivatives that are ubiquitous in plants (*i.e.* the so-called green leaf volatiles). These are low molecular weight compounds emitted in high concentrations by chopped *B. napus* but less so in whole *B. napus* leaves (data not shown). The initial EAG seen in response to *D. carota* extracts could be 1-hexanol because its retention time matches that of eluent A/B and because 1-hexanol is a known component of carrot volatiles (Guerin and Visser, 1980).

Dimethyl trisulfide, eluent E, evokes relatively small EAGs from *P. xylostella*. Sulfides are known stimulants of root maggots, *D. brassicae* and *D. antiqua*, especially

dimethyl disulfide (Guerin and Städler, 1982). Dimethyl disulfide and dimethyl trisulfide are major components of flower bud volatiles of *B. napus* (Tollsten and Bergström, 1988)

Monoterpenes generally do not appear to be detected by *P. xylostella* because no EAGs were observed with elutions of limonene, myrcene, pinene, and squalene, nor were EAGs observed with GC-effluent of the five-terpene standard. In non-GC-coupled EAG studies, Pivnick *et al.* (1994) fractionated 4-week old *B. juncea* extracts and found that *P. xylostella* produced EAGs to all fractions but gave large responses to one in particular (*i.e.* 'Fraction 6') which contained eight main compounds (borneol/isoborneol, 4-terpinenol, α-terpineol, verbenone, *cis*-carveol, naphthalene, 4-allylanisole, and benzothiazole). This fraction also attracted more moths in Y-tube assays and the authors suggested that one or more of these eight act synergistically with isothiocyanates in attracting moths. However, in GC-EAG studies described herein, moths did not respond to these compounds, and perhaps Fraction 6 of Pivnick *et al.* (1994) contained unreported, minor components that are stimulatory to *P. xylostella*. That is, the main compounds are not necessarily the active compounds as illustrated by EAG responses to eluents H and I.

No EAGs were observed in response to identified benzenoid compounds (naphthalene and benzaldehyde) except for eluent K, phenyl ethanol, which is a component of *Brassica* spp. flower fragrances (Tollsten and Bergström, 1988) and may be involved in location of food sources.

Benzothiazole and silicon are probably contaminants because these have not been reported in previous surveys of *Brassica* spp. volatiles (see Tollsten and Bergström,

1988; and Wallbank and Wheatley, 1976) and benzothiazole is a common contaminant of solvents (H.-R. Buser, personal communication).

Unfortunately, some peaks were not adequately resolved. Eluent G is comprised of at least two compounds (m/z = 112, 97 and 143, 73, 60) which inplies identification as a heptanol derivative and a C3 benzenoid. Eluent I is an aliphatic compound (m/z = 112, 98) and unidentifiable except as one of many hydrocarbons with a molecular weight of 112. Eluent H could not be resolved, in part, because of its small concentration in extracts and overlap with at least one neighboring peak (m/z = 91, 114 and 109, 124). However, neither eluent H nor eluent I can be an isothiocyanate because molecular weights and m/z numbers do not coincide with those of isothiocyanates (see Spencer and Daxenbichler, 1980).

No nitrogen-containing compounds, including isothiocyanates, were identified in *B. napus* extracts except for indole and phenyl acetonitrile, and these did not elicit EAGs from moths.

It is likely that constituent odors of *Brassica* spp. have the greatest influence on cruciferous insects as a blend of odors and, presumably, perception of the combined constituents reflect a host plant signature for identification. Necessary blends have been described for *C. assimilis* in which individual constituents elicited strong EAGs but were poor behavioral attractants unless combined (Evans, 1992). Also, EAGs only reflect the electrophysiological response which may manifest in positive, negative, or no apparent behaviors (Baur *et al.*, 1993).

Although EAGs provide some evidence that *P. xylostella* may be able to use olfaction to distinguish between *D. carota*, *S. alba*, and *B. napus* (glossy and waxy),

bioassays offered little information in this regard. *Daucus carota* volatiles apparently suppress egg deposition even when sinigrin (*i.e.* allyl glucosinolate) is present, but a lack of plant volatiles (*i.e.* paraffin oil only) has only minor consequences, if any. The rates at which eggs were deposited in the presence of *S. alba* and *B. napus* extracts were not significantly different and perhaps the resolution of this endpoint bioassay (*i.e.* egg counts after one and eight hours) is not optimal for determining behavioral differences in the presence of these various extracts. Likely, some low molecular weight volatiles, such as the leaf alcohols, were lost during the evaporation of hexane from artificial oviposition substrates, but 60 seconds was the minimum time in which the substrate could be sealed within the arena without moth mortality. Also, these assays offer only non-choice, closed systems whereas in field situations volatiles are not so discrete. If high molecular weight compounds do remain close to the leaf surface and serve as short-range stimulants, then a moth may abandon a host producing an 'inferior' blend and search out a leaf producing a more favorable blend of odors. Such a searching strategy is disallowed in bioassays of this study.

Also, different plant volatiles may affect adult *P. xylostella* in different ways; volatiles used in locating a host patch are likely to be long-range attractants, whereas volatiles used in oviposition site selection need not be long-range. Perhaps this is reflected in EAG dimorphisms of males and females, considering males may use host volatiles to locate sources of food and mates, whereas females need also to locate oviposition sites. Although bioassays in this study were somewhat inadequate, they did demonstrate that volatiles (especially those of non-host plants) affect oviposition. Females produced different EAG response patterns to *B. napus*, *S. alba*, and *D. carota*,

but whether volatiles influence oviposition site preferences for one crucifer over another (*e.g.* between glossy and waxy *B. napus*; Chapter 4) is unclear.

Table 5-I. EAG responses by male and female *P. xylostella* to GC-delivered isothiocyanate standards (mV ± standard error).

Moth sex	Isothiocyanates: allyl	butyl	phenyl
males (n = 5)	nm	0.92 ± 0.26^{a}	nr
females (n = 15)	0.97 ± 0.13^{c}	$2.26 \pm 0.18^{b,d}$	nr

nr = no response;
nm = response not reliably measurable.
[a,b] responses to butyl isothiocyanate; independent samples t-test, $\alpha = 0.05$, $P = 0.043$.
[c,d] responses by females; paired samples t-test, $\alpha = 0.05$, $P < 0.0001$.

Table 5-II. Compounds in headspace extracts of glossy *B. napus* identified by GC-MS.

Suggested identification	EAG response	Label	primary m/z .
Fatty acid derivatives:			
hexadienol	*	A	98, 83, 80
hexanol (+ others)	***	B	98, 83
hexenal	**	C	98, 97, 83
hexenol	**	D	100, 82
octynol	**	F	126, 111, 108
hexenyl acetate			
Benzenoids:			
phenol			94
benzaldehyde			106, 105
propanyl benzene			118, 117
phenyl ethanol	**	K	122, 91
naphthalene			128,127
benzothiazole			135, 108, 69
dichlorobenzene			148, 146
Terpenoids:			
limonene			93, 68, 67
myrcene			93, 69, 41
pinene			93, 41
squalene			37, 95, 93, 81
Nitrogenous compounds:			
indole			117, 90, 89
phenyl acetonitrile			117, 116, 90, 28
Others:			
silicon			
toluene			92, 91
dimethyl trisulfide	*	E	126, 111, 79
Unidentified:			
heptanol derivative? + C3 benzenoid	***	G	112, 97 (143, 73, 60)
unknown	****	H	112, 98
aliphatic compound	**	I	124, 109 (114)

numbers in brackets refer to m/z of a second compound

Table 5-III. Mean numbers of eggs per female (± standard error) deposited on artificial substrates in the presence of various headspace extracts; n = 6 for each extract.

Extract	Number of eggs deposited: in the first hour	during 8 hours
paraffin oil only	$0.50 \pm 0.34^{a,b}$	18.33 ± 3.20^{e}
B. napus waxy	5.50 ± 1.73^{c}	23.17 ± 2.50^{e}
B. napus glossy	5.33 ± 1.33^{c}	27.67 ± 2.73^{e}
S. alba	3.67 ± 1.12^{bc}	19.00 ± 2.30^{e}
D. carota	0.00^{a}	0.17 ± 0.17^{d}

[a,b,c] one-way ANOVA, $\alpha = 0.05$, $P = 0.002$; Duncan post hoc
[d,e] one-way ANOVA, $\alpha = 0.05$, $P < 0.0001$; Dunnett post hoc

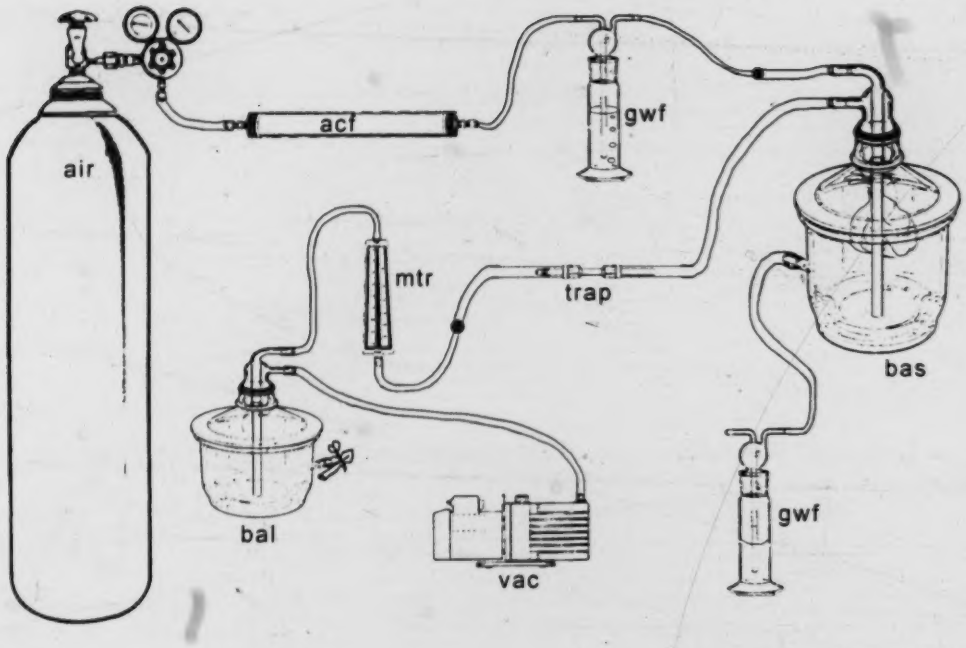

Figure 5-1. Push-pull airflow system (after Turlings *et al.*, 1990) for collection of volatiles of chopped plants. Compressed air (**air**) is directed through teflon tubing to an activated charcoal filter (**acf**), humidified at a gaswash flask (**gwf**) and passed into a glass basin with plant material (**bas**). Flow exits the plant basin to an adsorbent trap. Positive pressure is maintained and monitored with an attached gaswash flask. An in-line flow meter (**mtr**) monitored flow. An empty glass basin (**bal**) buffered minor fluctuations from the vacuum pump (**vac**). Black dots at tubing indicate teflon splitter/connectors where tandem plant basin, pressure monitor, and adsorbent trap were connected. (See text for details.)

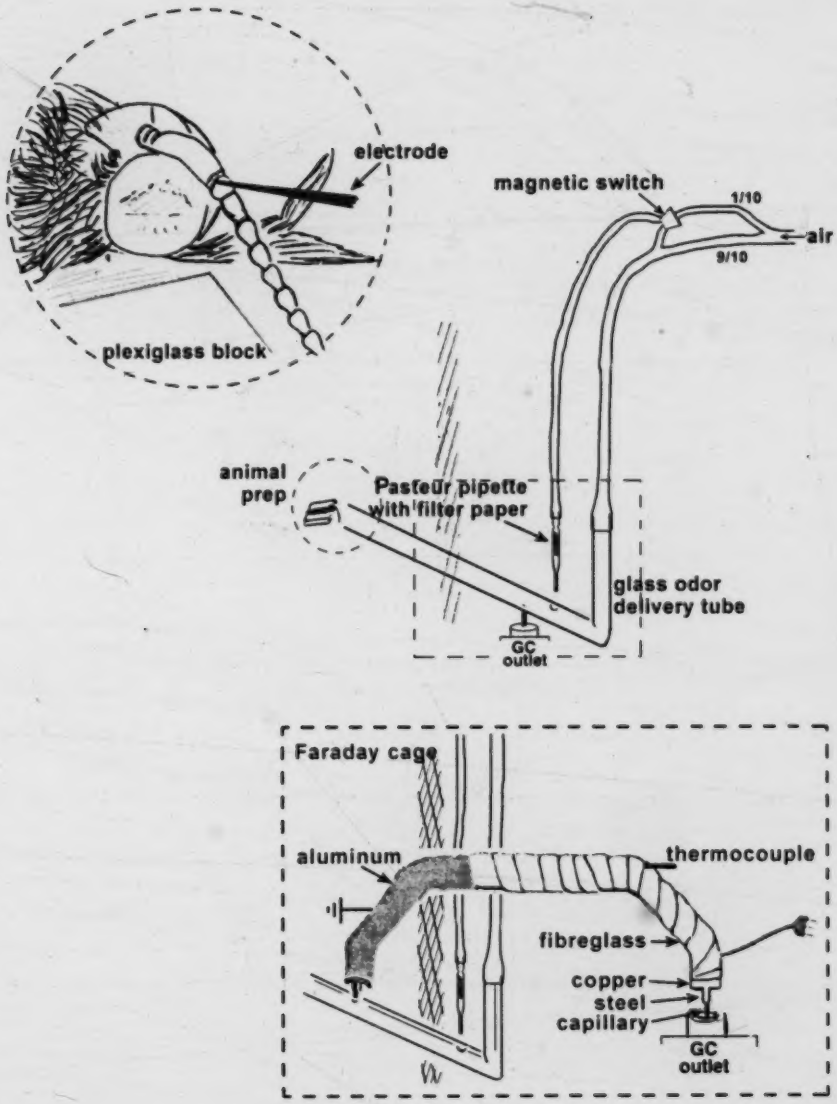

Figure 5-2. Odor delivery system to antennal preparation via GC and puff techniques. Circular inset is animal preparation with recording electrode inserted into base of antenna; rectangular inset is modified delivery system with shortened glass delivery tube. (See text for details.)

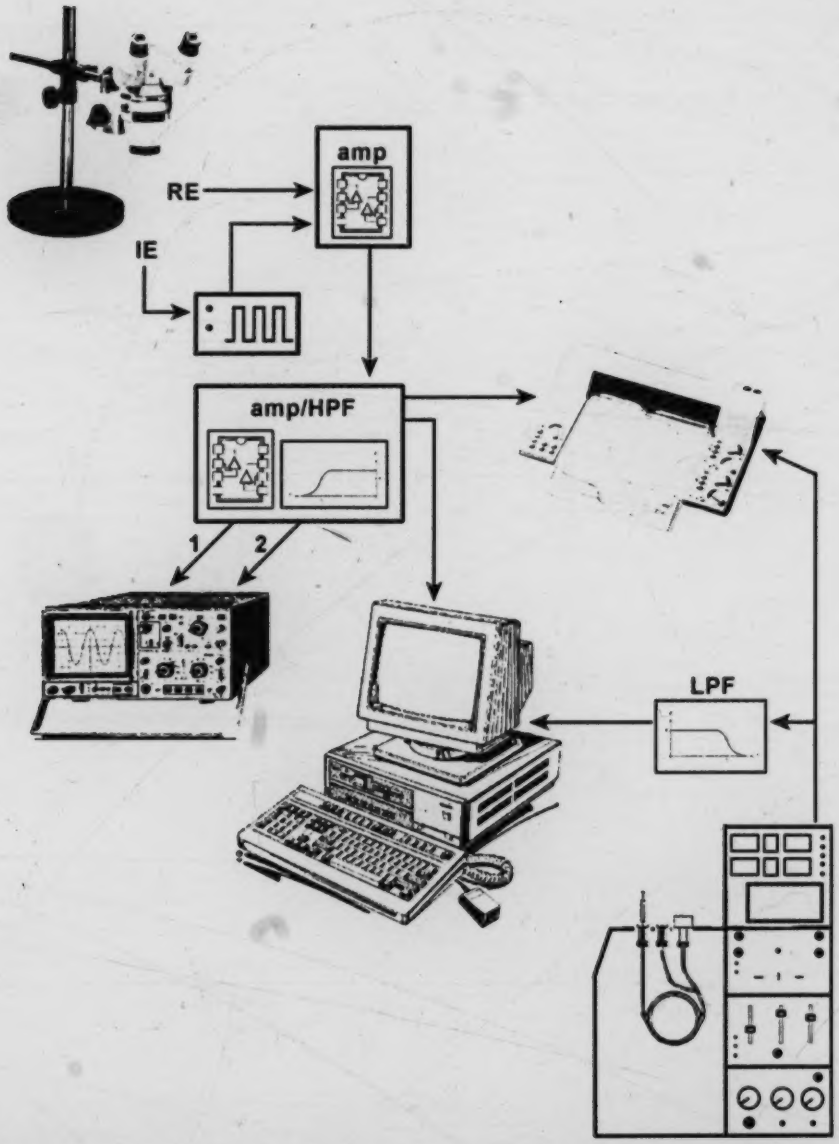

Figure 5-3. GC-EAG electronics setup. Indifferent electrode (**IE**) connected to a pulse generator which connected to a preamplifier as did the recording electrode (**RE**). The signal from the preamplifier was run through an amplifier and high pass filter (**HPF**); unfiltered (1) and filtered (2) signals were viewed on an oscilloscope. Unfiltered signal was sent to a computer; filtered signal to a two-pen chart recorder. GC signals were sent to the chart recorder and to the computer through a low pass filter (**LPF**). (See text for details.)

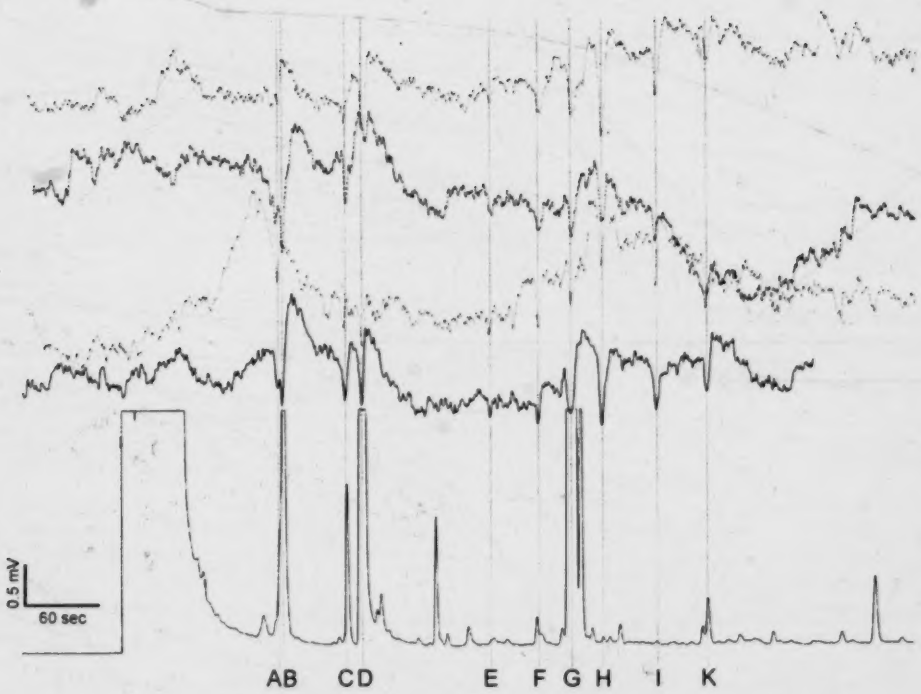

Figure 5-4. EAG traces from four female moths. Ten EAG peaks aligned with glossy *B. napus* FID trace. Letters designate eluents eliciting EAG responses; vertical scale bar refers to EAG traces; horizontal scale bar refers to EAG and FID traces.

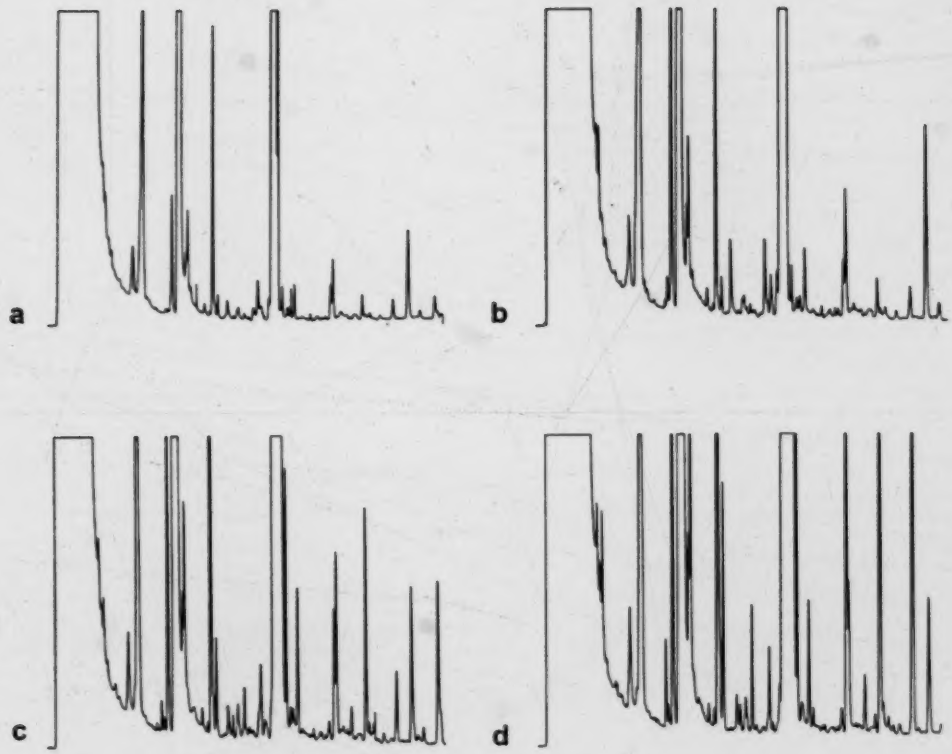

Figure 5-5. FID traces of *B. napus* headspace extracts: a) glasshouse-grown waxy; b) glasshouse-grown glossy; c) field-grown waxy; d) field-grown glossy.

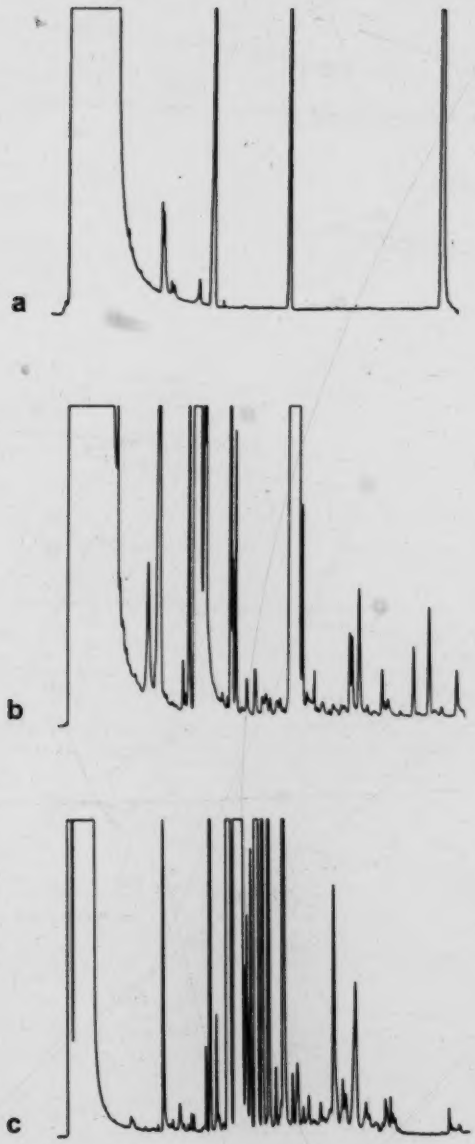

Figure 5-6. FID traces of: a) 0.1 mM isothiocyanate standard mixture; b) *S. alba* headspace extract; c) *D. carota* headspace extract.

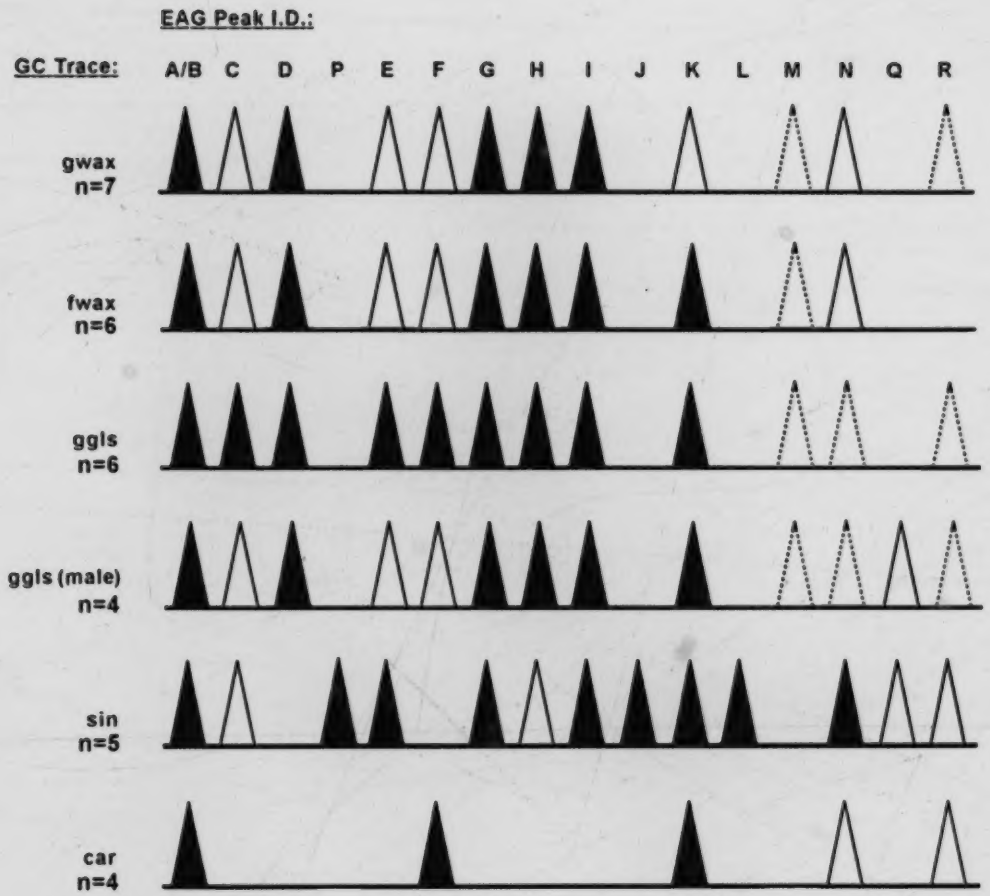

Figure 5-7. EAG peak identifications according to FID eluents. Black triangles are responses from >70% of animal preparations; white triangles from 30-70%; dotted triangles from 5-30%; missing triangles = 0%. All preparations were female except those designated 'mal'. Abbreviations: **gwax** = glasshouse-grown waxy; **fwax** = field-grown waxy; **ggls** = glasshouse-grown glossy; **sin** = *S. alba*; **car** = *D. carota.*

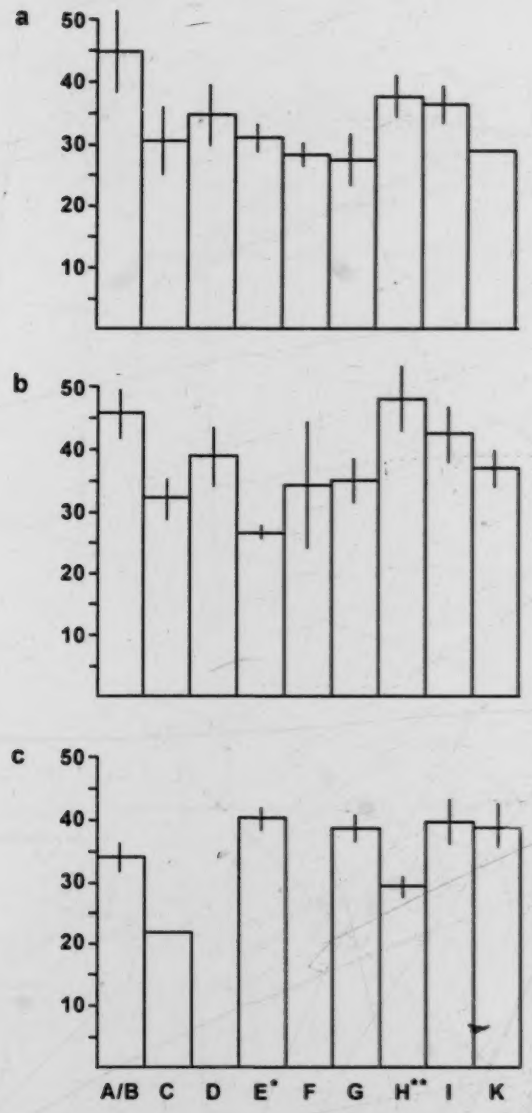

Figure 5-8. Arcsine-transformed responses (expressed as a percentage of response to butyl isothiocyanate) by female *P. xylostella* antennae to GC-delivered host plant extracts: a) waxy *B. napus*, b) glossy *B. napus*, c) *S. alba*. Capital letters correspond to FID eluents. Asterisks denote significant differences among plant extracts; two-way ANOVA; $\alpha = 0.05$, *P = 0.004; **P = 0.096.

Plate 5-I. 1 and 2) Scanning electron micrographs of *P. xylostella* antenna; bars = 100 µm and 10 µm, respectively; 3) ventral face of de-scaled antennal segment, bar = 20 µm; 4) dorsal face of de-scaled antennal segment, bar = 20 µm; 5) distal segment of antenna, bar = 20 µm; 6 and 7) perpendicular view of protuberance at apex, bars = 5 and 1 µm, respectively. Abbreviations: **cbs** - complete band of scales; **ch** - chaetic sensillum; **cv** - scale cavity; **d** - dorsal surface; **pbs** - partial band of scales; **pt** - protuberance; **st** - styloconic sensillum; **trI** - type I trichodeal sensillum; **v** - ventral surface.

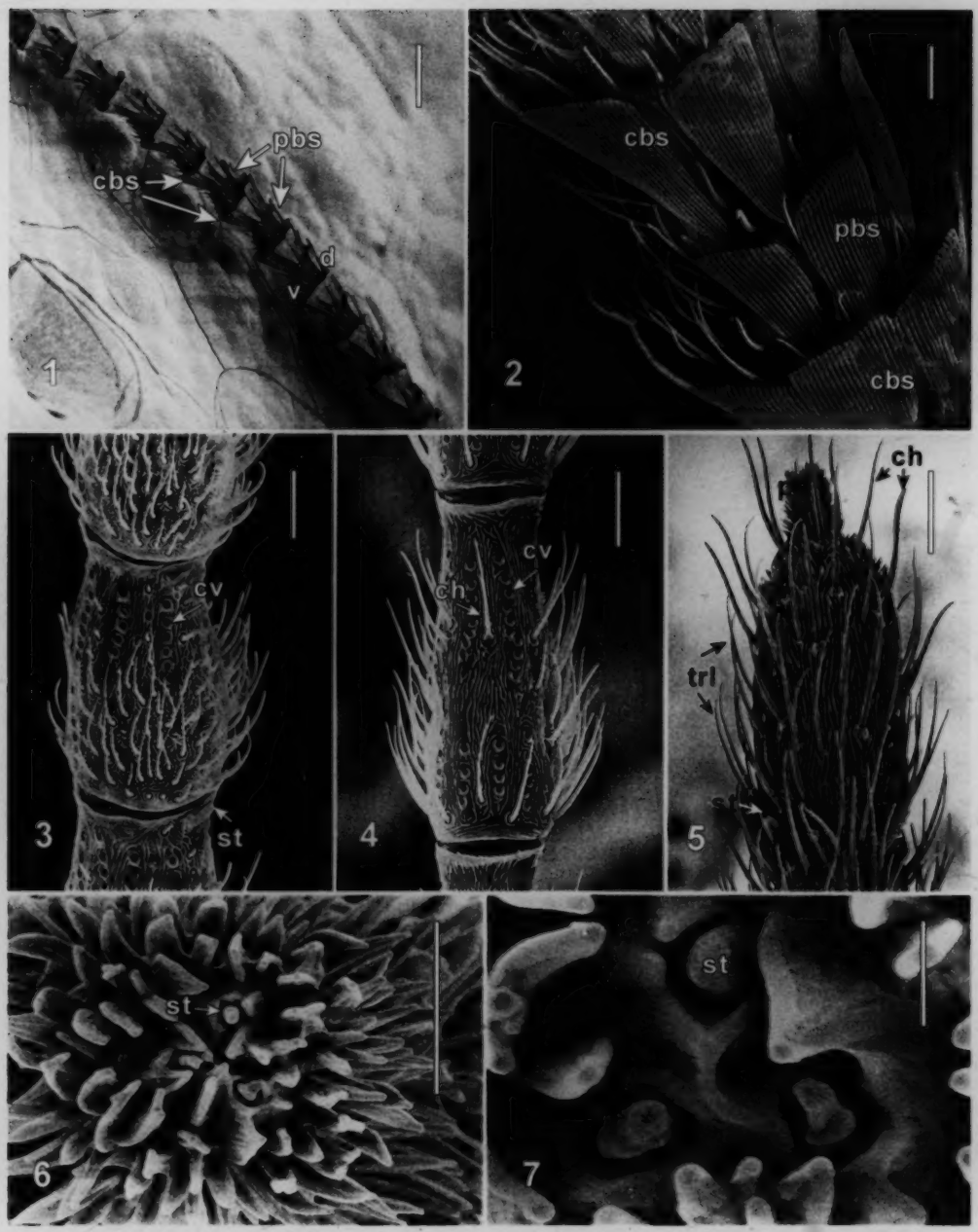
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Plate 5-II. 1) Scanning electron micrograph of antennal surface, bar = 10 μm; 2) socketed base of chaetic sensillum, bar = 1 μm; 3) tip of chaetic sensillum with five adducted fingers and sidewall terracing, bar = 500 nm; 4) styloconic sensillum at apical edge of segment, bar = 2 μm; 5) bud-shaped sensillum at apical edge of segment, bar = 1 μm; 6) coeloconic sensillum surrounded by cuticular spines, bar = 1 μm. Abbreviations: **bs** - bud-shaped sensillum; **ch** - chaetic sensillum; **cl** - coeloconic sensillum; **f** - cuticular finger; **is** – intersegmental membrane; **s** - cuticular spine; **sc** - scale; **sk** - socket; **st** - styloconic sensillum; **trI** - type I trichodeal sensillum; **trII** - type II trichodeal sensillum.

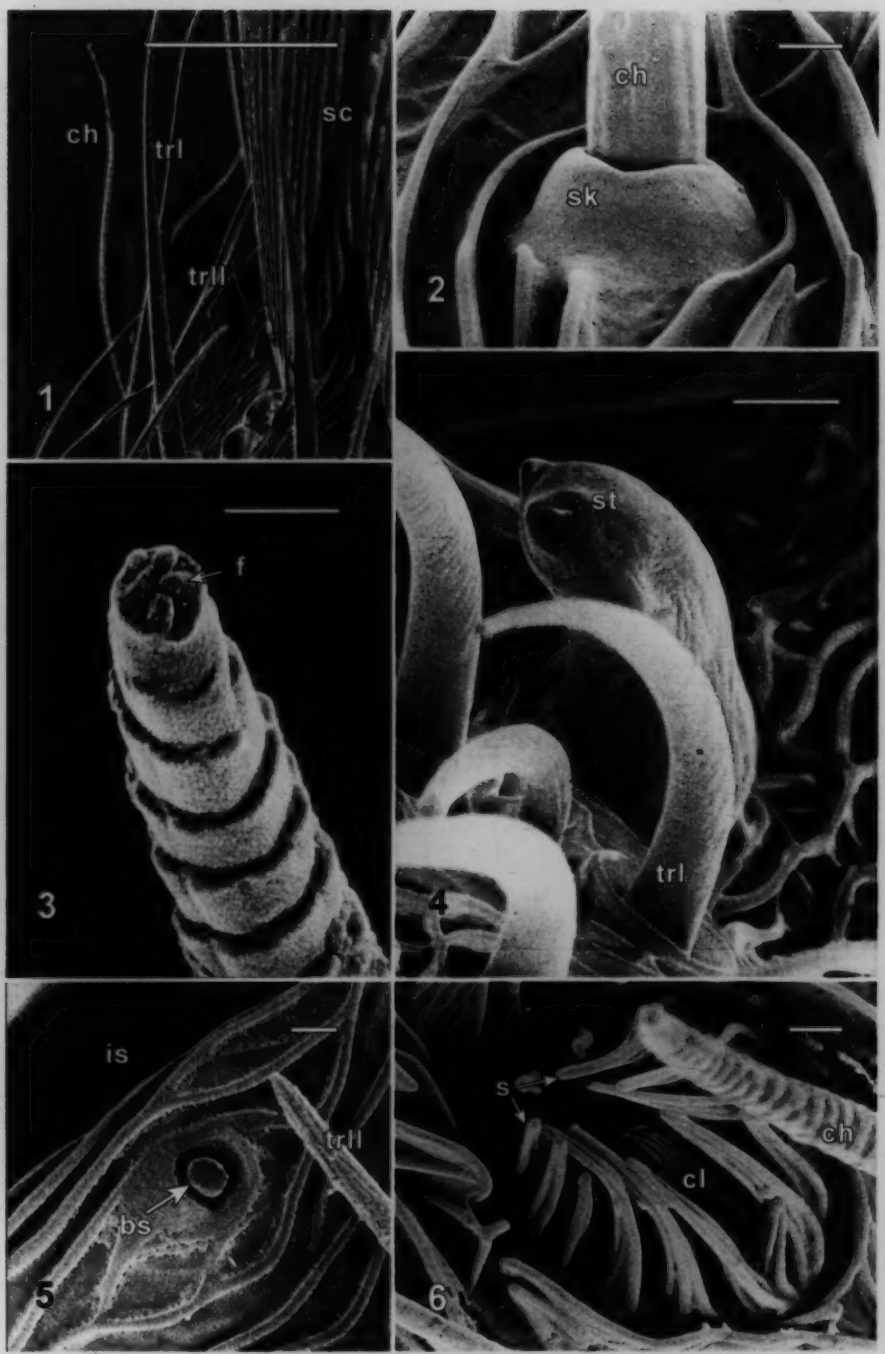
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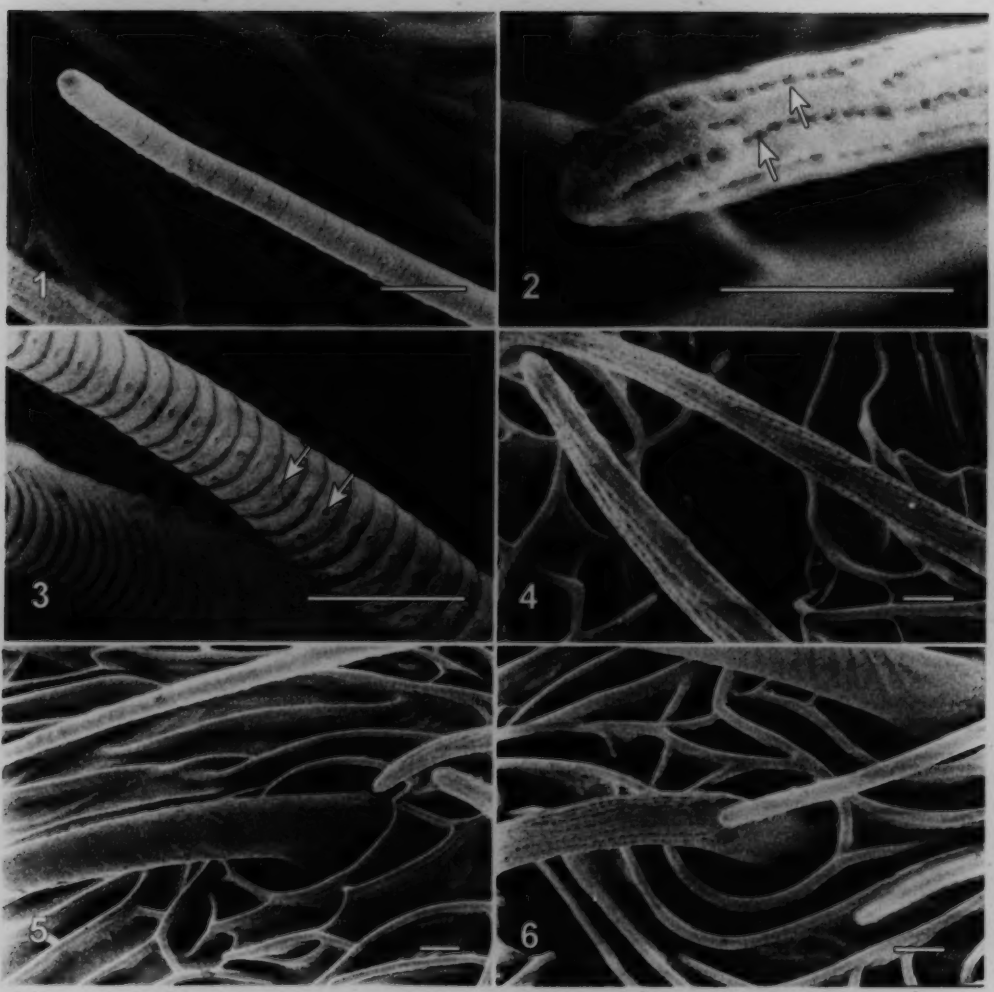
Plate 5-III. Scanning electron micrographs of trichodeal sensilla: 1) tip of type I; 2) tip of type II; 3) sidewall of type I; 4) sidewall of type II; 5) base of type I; 6) base of type II; bars = 1 μm. Arrows indicate pores.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5.5 Literature Cited

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Chapter 6:

Gustatory sensilla of *Plutella xylostella* and responses to surface extracts of *Brassica napus* and *Sinapis alba*.

6.1 Introduction

Gustatory (*i.e.* contact chemo-) sensilla are externally characterized by a single pore, usually at or near the tip, which may be surrounded by cuticular formations or fingers (Zacharuk, 1980). These sensilla are usually found on the mouthparts and tarsi of insects, but are also known to occur on ovipositors, antennae, and other body parts (Zacharuk, 1980; Städler, 1976). Dethier (1963) described the existence of mechano- , sugar- , salt- , and water-sensitive cells in gustatory sensilla of insects, and one or all of these can occur within one sensillum. Since then, other cells have been characterized, such as glucosinolate-sensitive cells (*e.g. D. floralis* tarsi, Simmonds *et al.*, 1994) and cells sensitive to amino acids (e.g. *Leptinotarsa decemlineata* Say, (Coleoptera: Chrysomelidae), Mitchell and Schoonhoven, 1974). Such functional classifications, although sometimes tenuous, have been used as evidence for the detection of compounds important in host plant acceptance.

Insects readily oviposit on substrates that possess appropriate general and specific compounds (*i.e.* 'sign stimuli') and reject substrates that possess deterrent compounds (Dethier, 1976). Sign stimuli are often perceived via gustation (Städler, 1984) and gustation is the ultimate step in identification of suitable diets and oviposition sites by insects (Ramaswamy, 1994; Schneider, 1987; Hodgson, 1968). Glucosinolates have been

implicated in stimulating oviposition by *D. floralis* (Simmonds *et al.*, 1994), *D. radicum* (Roessingh *et al.*, 1992a), *P. rapae* (Renwick *et al.*, 1992), and *P. brassicae* (van Loon *et al.*, 1992). Reed *et al.* (1989) reported the coincidence of glucosinolates in *Brassica* spp. homogenates and oviposition-stimulating activity of those homogenates for *P. xylostella.*

Plutella xylostella performs at least two behaviors, antennation and ovipositor sweeping (Chapter 3), that may facilitate gustatory sampling of potential oviposition sites. However, gustatory sensilla on the antennae, ovipositor, and other appendages may or may not possess cells involved in host plant recognition, and the nature of host plant stimuli involved has yet to be confirmed. To determine the location and activity of gustatory sensilla with respect to host identification, adult *P. xylostella* were surveyed for characteristic sensilla, and electrophysiological recordings from these sensilla were attempted.

6.2 Materials and Methods

6.2.1 Insects

Insects were reared at Vegreville, Alberta (under similar conditions described in Chapter 2) and pupae transported to facilities at Edmonton, Alberta. Adults used for electrophysiology were 3-5 days post-eclosion.

6.2.2 Scanning Electron Microscopy

Male and female moths, de-scaled via sonication and critical point dried, were surveyed for uniporous sensilla using either a Cambridge S-250 Stereoscan or a JSM-6301FXV field emission scanning electron microscope. (See Chapter 5 and Appendix III-1)

6.2.3 Plant extracts

Plants of glossy *B. napus* (S2 seed from Chapter 4) were grown in a glasshouse and plants of *S. alba* cv. Ochre grown in an environmental growth chamber. Glasshouse and growth chambers operated under similar conditions (22°C, 16L:8D). Plants were watered daily and fertilized (20:20:20) at the 4-leaf stage (growth stage 2.4; Harper and Berkenkamp, 1975). Leaves were harvested at stem elongation prior to flowering (growth stage 4.0; Harper and Berkenkamp, 1975). (*S. alba* plants showed relatively poor leaf development which was attributed to a malfunction in the light timers of the growth chamber.)

Leaves were dipped for five seconds each in dichloromethane, methanol, and deionized water, and were allowed to drip back into each solvent for three seconds before proceeding to the next. For each plant species, 250 g of fresh leaf material required 400 ml of each solvent creating extracts of 0.625 gram leaf equivalents (gle) per ml. (Approximate yields were 1200 ml of *B. napus* water extract and 400 ml of *S. alba* water extract).

Dichloromethane extracts were discarded. Methanol and water extracts were filtered under vacuum with medium crystalline filter paper (No. 1; Whatman International Ltd., England). Attempts to reduce methanol and a portion of *B. napus* water extracts were unsuccessful due to microbial contamination. The remaining water extract of *B. napus*, and all of the water extract of *S. alba* were filtered with 0.22 µm Sterifil D-GS low extractable filters (Millipore, USA) and stored at 4 °C until further use. Vacuum-filtered and double-filtered water extracts of *B. napus* were plated on potato dextrose agar (PDA) to explore microbial contaminants.

Stimulus solutions used for electrophysiology were made with combinations of KCl, sinigrin, and plant extracts (see Table 6-I for solutions and abbreviations; see Appendix I-9 for recipes). Conductivity of solutions was measured using a conductance meter (Model 35, YSI Scientific). Solutions were stored at 4 °C but allowed to warm to room temperature immediately preceding use.

6.2.4 Electrophysiology

Indifferent electrodes were made of filamented, thick-walled borosilicate glass capillaries (World Precision Instruments, Inc., USA), drawn using a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Company, USA) and filled with *Plutella* Ringer's solution (Appendix I-8).

Antennae were excised near a drop of Ringer's solution under a stereoscope so that the cut hydrophilic end of the antenna contacted the solution. Ringer's solution was drawn up adjacent to the antenna and a fresh cut was made, simultaneously drawing Ringer's solution into the new cut, to avoid potential effects of dessicated tissue from the original cut. A newly prepared indifferent electrode was inserted into the cut end of the antenna. To record from tarsal sensilla, similar preparations were attempted with forelegs but were not successful.

Recording electrodes were made from thin-walled borosilicate glass capillaries (Drummond Scientific Co., USA) drawn with an MI micropipette puller (Industrial Science Associates, Inc., USA) to yield a bore diameter that was slightly larger than the diameter of antennal sensilla. Recording electrodes were filled with KCl, sinigrin + KCl, or plant extract + KCl solutions (see Table 6-I for concentrations).

The recording electrode was attached to a high input impedence (10^{-15} Ω) preamplifier (P.J. Albert, Canada). This, and the indifferent electrode, connected to a clamping amplifier (G. Johnson, USA) that had been modified to accomodate the preamplifier. Differential electrical signal was sent to an amplifier/filter (high pass cornering frequency of 3 kHz; low pass cornering frequency of 1 Hz), and both unfiltered and filtered signals were monitored on a dual beam oscilloscope. Filtered signal was recorded on a mini-disk digital recorder (JA3ES all band 20bit sampling rate converter, Sony, USA) along with a vocal record of preparation and stimulus details on an audio track. A computer, equipped with AutoSpike (Syntec NL, the Netherlands) and SAPID Tools software (Smith *et al.*, 1990), displayed and analyzed digital information (Fig.6-1).

6.3 Results

6.3.1 Scanning Electron Microscopy

Uniporous sensilla were observed on the antennae (Chapter 5), proboscis, tarsi, and ovipositor, but not on labial palps.

Styloconic sensilla (approx. 1 μm in diameter) of the proboscis were set on notched angular bases of variable heights (range 3.0 to 7.5 μm) (Plate 6-I.1 and 2). These existed only on the ventral surface of the proboscis such that when the proboscis is not extended, they are protected within its coil.

Seven sensilla were arranged symmetrically on the tarsus, with one between and one beneath each of the two tarsal claws and the remaining four evenly dispersed around the anterior of the arolium. These sensilla were similar to chaetic sensilla of the antennae

(see Chapter 5) with a length of approximately 36 μm and a pore approximately 120 nm in diameter surrounded by five cuticular fingers (Plate 6-I.3 and 4).

Each lobe of the ovipositor had a longitudinal row of four to six uniporous hairs along the margin of the ventral midline where left and right lobes meet (Plate 6-I.4). These chaetic sensilla (approx. 30 μm) were socketed but, unlike antennal and tarsal sensilla, lacked cuticular fingers and sidewall terracing (Plate 6-I.5 and 6). Numerous short (approx. 15 μm) and long (approx. 45 μm) aporous hairs covered the ovipositor but only the shorter sensilla appeared between the two rows of uniporous sensilla (Plate 6-I.5).

6.3.2 Electrophysiology

Potato dextrose agar streaked with vacuum-filtered and double-filtered extracts did not support any microbial growth.

Conductances of stimulating solutions are given in Table 6-I.

A large percentage of sensilla responded to 250 mM KCl and responses (spikes/second) to KCl were greatest at 250 mM (Fig. 6-2) for both males and females (ANOVA, $\alpha = 0.05$, $P < 0.0001$). Also, sensilla on male antennae were more sensitive to KCl concentrations than female antennae (ANOVA, $\alpha = 0.05$, $P = 0.002$) (Fig. 6-2, a).

Because a number of sensilla showed no response to 100 mM and 500 mM KCl, comparisons were made between 'KCl-responders' (those that responded to KCl) and 'non-responders' (those that did not respond to any concentration of KCl) when these sensilla were stimulated with *B. napus* and *S. alba* extracts. In both sexes, KCl-responders produced more spikes/second to plant extracts with 100 mM KCl than did

KCl-non-responders (Table 6-II, Fig. 6-3). However, extracts with 500 mM KCl evoked similar responses from responders and non-responders (Table 6-II).

For KCl-responders, responses to 0.625 gle *B. napus* + 100 mM KCl (0.625Bn100KCl) were nearly double those to 100 mM KCl alone, and responses to 0.625 gle *S. alba* + 100 mM KCl (0.625Sa100KCl) were significantly greater than those to 0.625Bn100KCl (Fig. 6-4). Combining 0.625Bn100KCl with 0.625Sa100KCl in a 50:50 ratio did not provide a better response than 0.625Sa100KCl alone (Fig. 6-4).

Few sensilla responded to KCl-deficient plant extracts (0.625Bn0KCl and 0.625Sa0KCl) (Table 6-I) and those that did produced very poor responses (1.78 ± 0.38, and 4.77 ± 1.62 spikes in 700 msec, respectively). Equally poor responses were observed with 10 mM sinigrin plus 100 mM and 500 mM KCl (Table 6-I).

Although not significantly different from other *B. napus* concentrations, 0.3125Bn100KCl and 0.625Bn100KCl elicited maximal response; applications of extracts with higher and lower gle produced fewer spikes per second (Fig. 6-5).

6.4 Discussion

Sensilla on the proboscis of *P. xylostella* are morphologically identical to those of *C. fumiferana* (Städler, 1984) which possess a sugar-sensitive and (perhaps) a water-sensitive cell (Städler and Seabrook, 1975). *Plutella xylostella* rarely extend their proboscis during oviposition (Chapter 3) and it is probable that these styloconic sensilla are used exclusively in feeding, as is the case with most mouthpart-born sensilla (Städler, 1978). Because of this, and problems encountered with preparation setups, no attempts were made to record from these sensilla.

Uniporous sensilla have been reported on the tarsi of many Lepidoptera (*e.g. Danaus plexippus*, (Danaidae) Baur *et al.*, 1998; *Papilio polyxenes*, (Papilionidae) Roessingh *et al.*, 1991; *P. brassicae*, Ma and Schoonhoven, 1973). Those of *P. xylostella* are at least superficially similar to the chaetic sensilla of its antennae. A similar condition was noted in *E. kühniella*, but also included innervation patterns of four chemo- and 1 mechanosensory cell in both tarsal and antennal sensilla (Anderson and Hallberg, 1990).

Tarsal chemosensilla of *P. xylostella* are the first to contact the leaf surface and may respond to epicuticular phytochemicals, but attempts to record from these sensilla were unsuccessful due to inadequate tarsal mounts. (Insertion of the glass capillary perturbed the arrangement of tarsomeres such that tarsomeres adducted and made contact with a recording electrode extremely difficult.) In other cruciferous insects, at least one cell innervating tarsal contact chemosensilla appears to be receptive to glucosinolates (*D. floralis*, Simmonds *et al.*, 1994; *D. radicum*, Roessingh *et al.*, 1992a; *P. brassicae*, Ma and Schoonhoven, 1973). Further, different glucosinolates evoke different responses from the same sensillum of *D. floralis* and *D. radicum* (Simmonds *et al.*, 1994; Roessingh *et al.*, 1992a; respectively) indicating either that innervating cells possess distinct receptors for various glucosinolates or, more likely, that receptors have higher binding affinities for some glucosinolates. In any case, sensory cells can promote specificity even among members of a chemical genus and may function in host plant choices by ovipositing females.

Ovipositor sensilla of *O. nubilalis* (Marion-Poll *et al.*, 1992), *E. kühniella* (Anderson and Hallberg, 1990), and *Helicoverpa armigera* (Baker and Ramaswamy, 1990) are of two types: aporous mechanosensory and uniporous gustatory hairs

(determined with electron microscopy and/or electrophysiology), and these vary among species in number and location. Ovipositors of *P. xylostella* also possess aporous and uniporous sensilla. These uniporous sensilla respond to sinigrin in a dose-dependent manner (Mitchell, unpublished data) which agrees with findings by Reed *et al.* (1989) in which *P. xylostella* oviposited on filter paper treated with *B. juncea* homogenate fractions that contained sinigrin.

Whereas tarsal and ovipositor sensilla are obvious candidates in many insects for host detection mechanisms because these body parts contact substrate surfaces, antennae are not especially obvious because most insects do not contact host surfaces with their antennae. Until recently, antennal gustatory sensilla have been considered to function primarily in mate recognition via contact pheromone receptors, and the involvement of antennal contact chemosensilla in plant-insect interactions has been neglected (Städler, 1976). Baur *et al.* (1998) reported that contact chemosensilla on the antennae and tarsi of *D. plexippus* are highly receptive to fractions of host plant extracts (at 0.1 and 1.0 gle) and similar fractions stimulated oviposition by *D. plexippus* (Haribal and Renwick, 1996). Likewise, *P. xylostella* strike the substrate with their antennae during oviposition bouts which implies a gustatory involvement in host plant detection and oviposition site selection (Chapter 3), and antennal sensilla respond to host plant extracts (at least as low as 0.125 gle).

Attempts to show a dose-response relationship for extracts of *B. napus* in antennal sensilla were somewhat unsuccessful due to the technique employed in concentrating extracts. In most cases, the stimulus artifact during recording was relatively small and conductance of 0.125 gle and 2.5 gle were similar. Because extract was only partially

reduced (Appendix I-9), increased viscosity may have interfered with recording electrical potentials. This problem could be remedied by reducing extracts to dryness and rehydrating to appropriate concentrations. However, dilutions of extracts did allow the delineation of the upper slope of a dose-response curve in which 0.3125 gle appears to produce maximal spike frequency. Further dilutions are neccessary to determine thresholds.

Several explanations could account for the lesser response to *B. napus* than to *S. alba* extracts. Firstly, gle is only a relative unit measurement and provides no information about absolute concentrations of extract components. If the stimulatory component in *B. napus* is the same as that in *S. alba*, the response differences may be due to unequal stimulus concentrations, and this is supported by stronger responses to 0.625Sa100KCl and Comb100KCl than to 0.625Bn100KCl. Leaves of *S. alba* were smaller than those of *B. napus* and therefore more leaves were required to make up 250 g, (approximately 1.5 times that of *B. napus*) which may have resulted in higher concentrations within *S. alba* extracts even though both extracts were 0.625 gle.

Secondly, if the response is due to glucosinolate content, response differences could be due to quantity and/or quality of glucosinolates within *B. napus* and *S. alba* extracts. Cultivars used in this study were of low- (*B. napus*) and high-glucosinolate (*S. alba*) content in seed, which may or may not equate to differences in glucosinolate quantity in seed, but it is highly unlikely that a single glucosinolate occurred in both plant extracts. It is also unlikely that the stimulatory component in either extract is sinigrin (*i.e.* allyl glucosinolate) because sinigrin is not known to occur in *B. napus* or *S. alba* (Reed *et al.*, 1989) and because sinigrin with either 100 or 500 mM KCl produced responses

comparable to those of 100 and 500 KCl only. Sinalbin (*i.e.* p-hydroxybenzyl glucosinolate) occurs exclusively in *S. alba* (Bodnaryk, 1991; Reed *et al.*, 1989), whereas indole glucosinolates are found *in B. napus* (Bodnaryk, 1997; Reed *et al.*, 1989). Further, receptor specificity such as that found in tarsal sensilla of *Delia* spp. (Simmonds *et al.*, 1994; Roessingh *et al.*, 1992a) may be a factor in disparate responses to these two extracts.

Responses by antennal sensilla may not be due to glucosinolate-sensitive cells. In fact, the idea that glucosinolates are not solely responsible for attracting cruciferous insects is gaining recognition and the evidence is compelling. Non-polar fractions of *B. oleracea* are highly stimulatory to ovipositing *P. xylostella* (Hughes *et al.*, 1997) and receptor cells of *D. radicum* tarsi are highly sensitive to 'CIF', a non-glucosinolate compound isolated from surface extracts of *B. oleracea* (Roessingh *et al.*, 1992b.)

Städler and Roessingh (1990) speculated that microbes may play a role in plant-insect interactions. No microbial colonies were observed with potato dextrose agar when extracts were plated on this medium, and it is unlikely that any microbes directly influenced sensillar responses. This does not preclude the existence of microbial products in plant extracts, but it is improbable that these products would be ubiquitous in all host plant patches, or that preferences among crucifers would universally depend on the presence of specific microbial products.

Fractionation and analysis of *B. napus* and *S. alba* surface extracts for continued use in electrophysiology and bioassays would provide a better understanding of required sensory input. If a single factor within plant extracts (glucosinolate or not) is responsible for eliciting electrophysiological responses, there are several conceivable cellular

mechanisms within a sensillum: a single cell responds to both KCl and plant extract, one cell responds to KCl (*i.e.* a 'salt cell') and one cell responds to some compound in the plant extract (*i.e.* a 'plant cell') and these two cells act independently, the salt cell inhibits the plant cell, the salt cell is inhibited by the plant cell, or one cell promotes the other.

1. A single cell responds to both salt and plant extract. This is the most parsimonious of all possibilities and, because spike size and shapes appear to be homogeneous without the occurrence of 'doublets' (*i.e.* two spikes that overlap in time to create one large spike or a spike with a double peak in the recorded spike-train), the observed response to KCl and plant mixtures appear to represent the activity of a single cell. However, that a greater proportion of sensilla responded to plant extracts plus KCl, and with greater spike frequency than KCl only, indicates that either the plant extract simply contains salt and the salt cell fires with greater frequency, or receptors for salt and plant extract occur in the same cell and these interact to produce the observed responses, or the plant cell is different from the salt cell.

It is unlikely that the response elicited by plant extract is simply a response to salt content within the extract because conductance of plant extracts is quite poor (< 1.0 millisiemens) compared to 50 mM KCl (4.3 millisiemens). Also, extract without the addition of KCl elicits few responses and with frequencies below that of 50 mM KCl, and responses are not additive (*i.e.* spike frequency in response to 100 mM KCl less spike frequency to 0.625Bn100KCl by KCl-responders does not equal the spike frequency to 0.625Bn100KCl by non-responding sensilla, and the 0.625Bn100KCl response in KCl-responding sensilla should be less than the 250 mM KCl response).

Interactions between salt and components of plant extracts within one cell are quite possible. Salt and plant extract constituents could bind cooperatively to one receptor (accounting for the increased spikes/second to plant extract with KCl versus plant extract or KCl alone), or salt and plant extract receptors may be separate but influence each other via second messenger systems. (For example, transduction in vertebrate olfactory receptors occurs via activation of G-proteins by receptor-bound odorant molecules which, in turn, activate cAMP to open cation channels (J. Goldberg, personal communication)).

2. The salt cell and the plant cell fire independently. This possibility can be dismissed because of the lack of doublets and because plant extract with 100 mM KCl does not evoke responses that are equal to the summed responses of 100 mM KCl alone and plant extracts alone.

3. The salt cell inhibits the plant cell. While this may be true at high concentrations (*i.e.* 500 mM KCl), it is not the case at lower concentrations because the response to plant extracts with 100 mM KCl is greater than the response to 100 mM KCl alone or plant extract alone.

4. The salt cell is inhibited by the plant cell. If this were true, it is likely that responses to plant extracts with 100 mM KCl would be similar to plant extracts with 500 mM KCl in sensilla that normally respond to KCl. Except for the lack of doublets, data support this hypothesis.

5. One cell promotes the other. Data also support this possibility in that plant extracts with100 mM KCl evoke a response greater than the sum of responses to plant extract alone and 100 mM KCl alone.

It does not seem possible that these last two mechanisms could co-occur because inhibition of the salt cell should disallow its ability to provide feedback to enhance the plant cell and, obviously, the plant cell could not simultaneously enhance and depress the salt cell. Of course, the involvement of more than two cells and/or the presence of more than one stimulus in a plant extract is reasonable, but it would be difficult to account for the lack of doublets in spike trains. Further, although sensilla differed in their responses to salt, which allowed the classification and separation of KCl- responders and non-responders during analysis, no other obvious differences were found. Other variabilities among sensilla may exist and their discovery would aid in distinguishing the number of stimuli and cells involved. Clearly more work is required to elucidate the role of contact chemosensory cells in *P. xylostella* oviposition. What is salient is the extremely poor response to sinigrin, a compound that has been well established as an oviposition stimulant for this insect species.

Table 6-I. Abbreviations and conductivity of stimuli used for tip-recording from uniporous antennal sensilla of *P. xylostella*

Stimulus (abbreviation)	Conductivity*	No. of sensilla tested		Response
		males:	females:	
50 mM KCl (50KCl)	4.30	15	15	+
100 mM KCl (100KCl)	10.67	89	73	++
250 mM KCl (250KCl)	22.80	26	22	+++
500 mM KCl (500KCl)	43.30	27	45	++
10 mM sinigrin + 100 mM KCl (Sin100KCl)	9.69	2	5	nr
10 mM sinigrin + 500 mM KCl (Sin500KCl)	30.70	10	26	+
2.5 *B. napus* + 100 mM KCl (2.5Bn100 KCl)	13.67	0	3	+++
1.25 *B. napus* + 100 mM Kcl (1.25Bn100 KCl)	12.96	3	5	+++
0.625 *B. napus* + 0 mM KCl (0.625Bn0 KCl)	0.63	10	9	+
0.625 *B. napus* + 100 mM KCl (0.625Bn100 KCl)	8.08	68	56	+++
0.625 *B. napus* + 500 mM KCl (0.625Bn500 KCl)	18.58	38	49	+++
0.3125 *B. napus* + 100 mM KCl (0.3125Bn100 KCl)	9.75	4	7	+++
0.125 *B. napus* + 100 mM KCl (0.125Bn100 KCl)	10.46	4	6	+++
0.625 *S. alba* + 0 mM KCl (0.625Sa0 KCl)	0.82	13	4	+
0.625 *S. alba* + 100 mM KCl (0.625Sa100 KCl)	7.37	40	32	+++
0.625 *S. alba* + .625 *B. napus* + 100 mM Kcl (Comb100 KCl)	7.55	31	25	+++

* Conductance measured in millisiemens; conductance of deionized H_2O = 0.0160 millisiemens

\+ - less than 20% sensilla responding; ++ - approximately 50% responding; +++ - greater than 80% responding; nr - no response.

Table 6-II. Responses to *B. napus* (**Bn**) and *S. alba* (**Sa**) extracts plus 100 mM and 500 mM KCl by sensilla that did and did not respond to comparable KCl solutions without plant extracts. (See Table 6-I for abbreviation definitions.)

	Sensillar response ± standard error (and sample size)		
Stimulus:	KCl-non-responder	KCl-responder	*P*-value**
males:			
100KCl	---	13.00 ± 1.45 (36)	---
0.625Bn100KCl	10.61 ± 1.50 (33)	20.53 ± 2.35 (32)	0.001
0.625Sa100KCl	14.30 ± 1.36 (27)	25.70 ± 5.90 (10)	0.01
500KCl	---	7.33 ± 1.55 (18)	---
0.625Bn500KCl	13.33 ± 3.61 (16)	17.80 ± 2.50 (15)	ns
females:			
100KCl	---	7.83 ± 0.88 (34)	---
0.625Bn100KCl	8.86 ± 1.78 (22)	21.12 ± 2.70 (25)	0.001
0.625Sa100KCl	16.63 ± 2.90 (16)	30.50 ± 5.92 (11)	0.028
500KCl	---	4.55 ± 1.55 (18)	---
0.625Bn500KCl	12.95 ± 1.22 (22)	16.50 ± 3.26 (17)	ns

* spikes in 700 msec

** Independent samples t-tests, $\alpha = 0.05$.

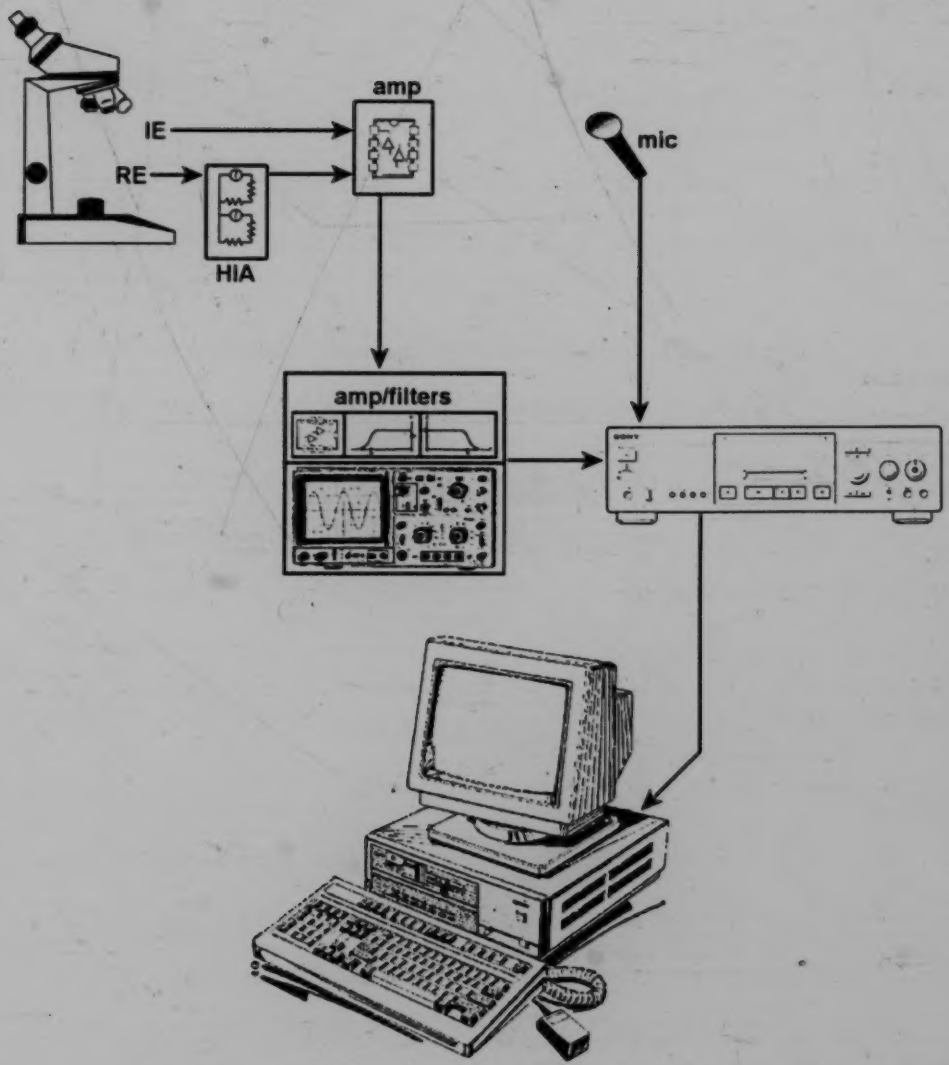


Figure 6-1. Tip-recording electronics setup. Recording electrode (**RE**) connected to a high input impedence preamplifier (**HIA**) which, along with the indifferent electrode (**IE**), connected to a modified clamping amplifier. The signal was amplified and filtered; both unfiltered (1) and filtered (2) signals were monitored on an oscilloscope. Vocal tracks and filtered signals from the antennal preparation were recorded on a Sony mini-disk recorder. Digitized data was transfered to a computer for analysis. (See text for details.)

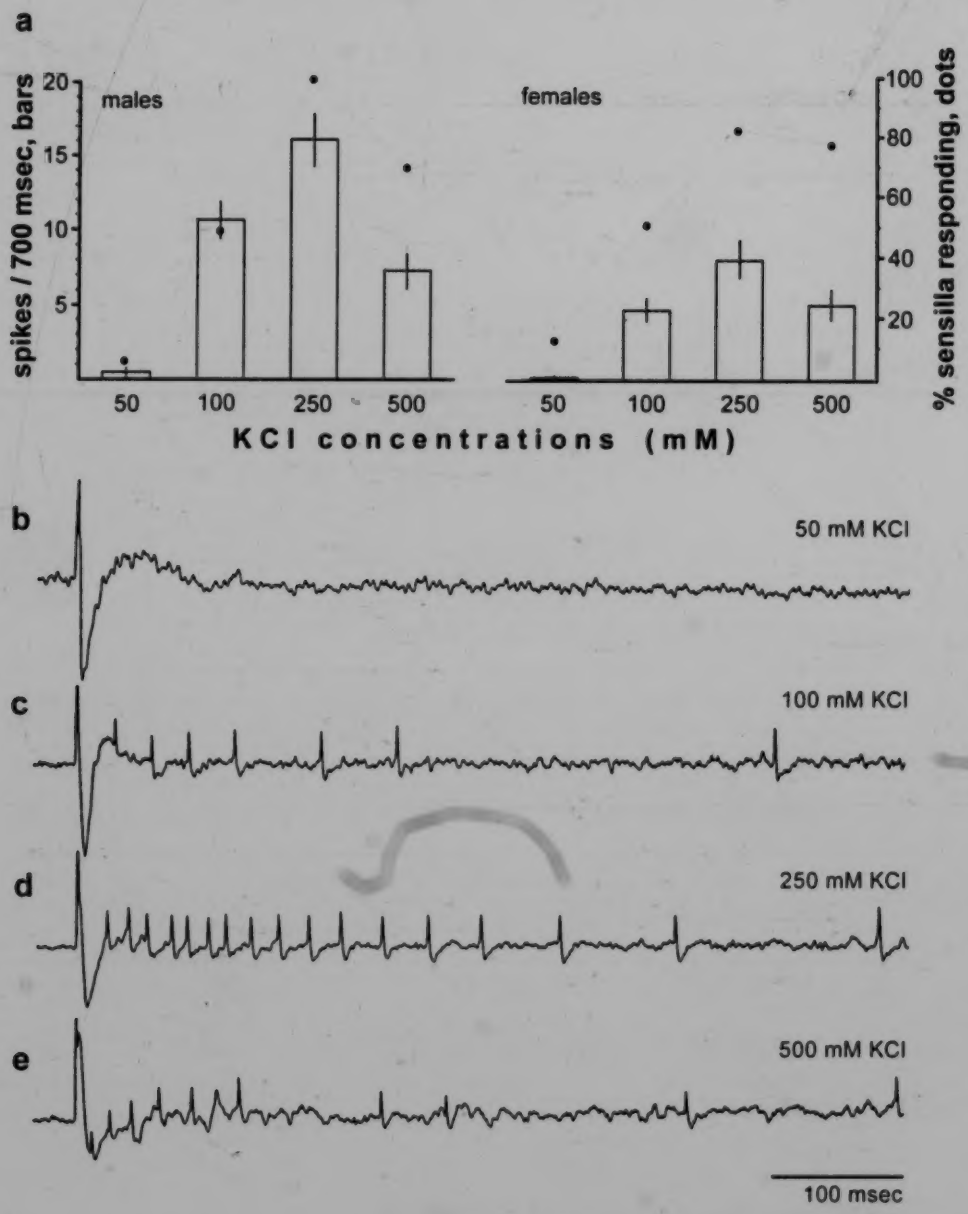


Figure 6-2. a) Responses to four KCl concentrations by males and females; bars (left axis) are number of spikes recorded in the first 700 msec post-stimulation ± standard error; dots (right axis) are percentage of sensilla responding; sample sizes are given in Table 6-I. b - e) recordings from one sensillum on the distal segment of a female antenna.

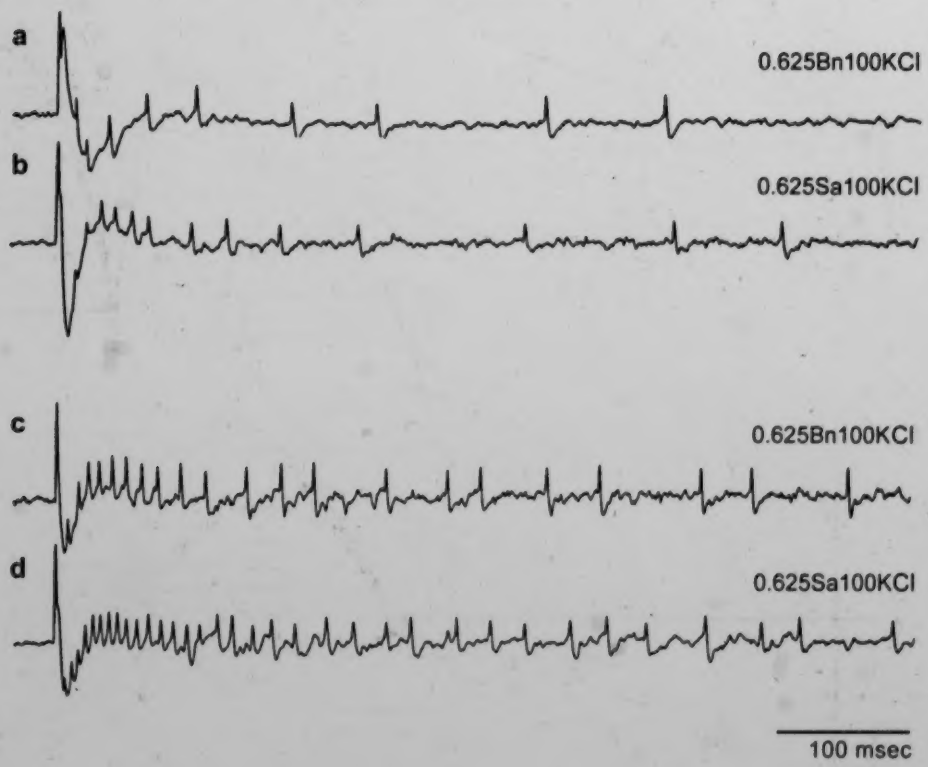


Figure 6-3. Tip-recordings from (a and b) KCl-non-responders and (c and d) KCl-responders to *B. napus* (**Bn**) and *S. alba* (**Sa**): extracts a) 0.625Bn100KCl, b) 0.625Sa100KCl, c) 0.625Bn100KCl and, d) 0.625Sa100KCl. (See Table 6-I for abbreviation definitions.)

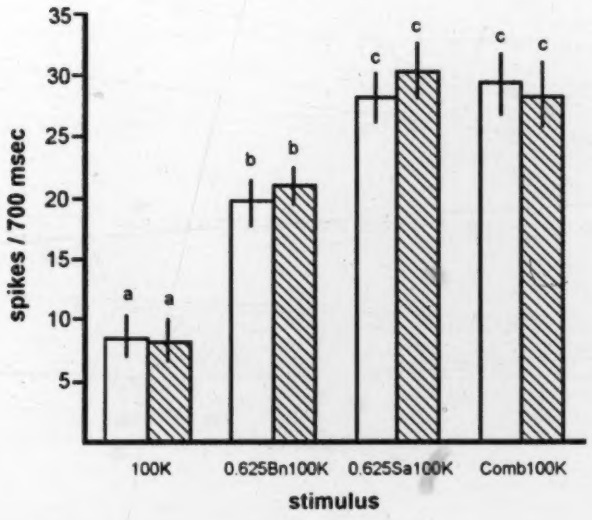


Figure 6-4. Number of spikes ± standard error within 700 msec post-stimulation recorded with four stimulus solutions: 100KCl (n = 19 males, 21 females), 0.625Bn100KCl (n = 24 males, 20 females), 0.625Sa100KCl (n = 40 males, 32 females), and Comb100KCl (n = 29 males, 22 females). Male responses are open bars, female responses are hatched bars. Letters denote significant differences (one-way ANOVA, $\alpha = 0.05$, $P < 0.0001$).

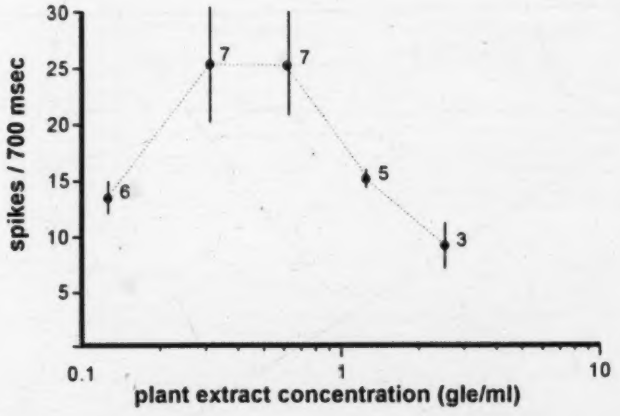
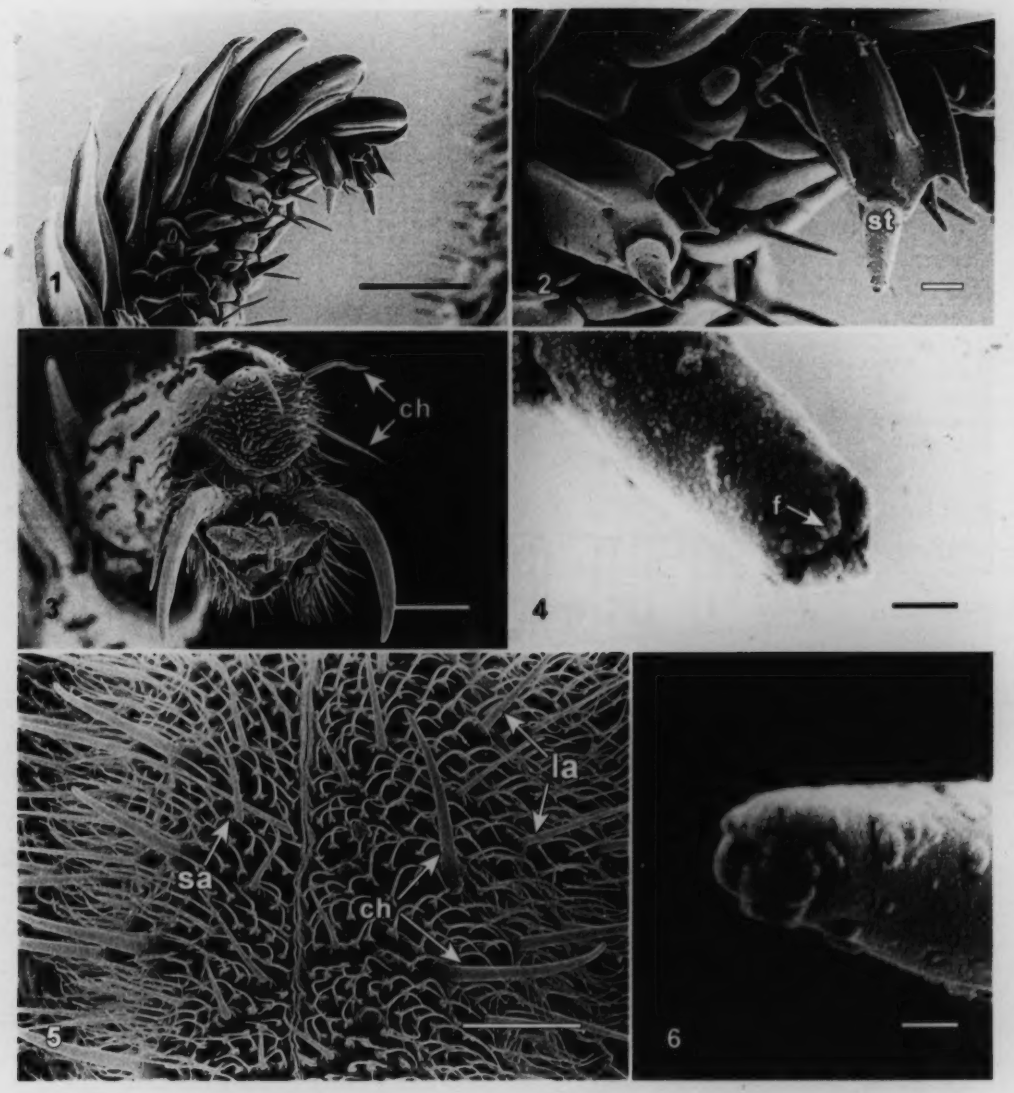


Figure 6-5. Responses of *B. napus* surface extracts with 100 mM KCl. Each data point represents mean response ± standard error; sample sizes for each extract concentration are given beside respective data points.

Plate 6-I. Scanning electron micrographs of : 1 and 2) proboscis with styloconic sensilla, bars = 10 and 1 μm, respectively; 3) fore-tarsus, bar = 20 μm; 4) tip of fore-tarsal sensillum, bar = 200 nm; 5) ovipositor, bar = 20 μm; and 6) tip of ovipositor uniporous sensillum, bar = 200 nm. Abbreviations: **ch** - chaetic sensillum, **f** - cuticular finger, **la** - long aporous sensillum, **sa** - short aporous sensillum, **st** - styloconic sensillum.


1
2
st
ch
3
f
4
la
sa
ch
5
6

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Chapter 7:

General Discussion

7.1 Conclusions

There are remaining gaps in our knowledge of *P. xylostella* biology, not the least of which is whether this is a single species exhibiting reproductive (*e.g.* lack of spermatophore formation) and morphological (*e.g.* degree of sclerotization of the bursal duct) variation among populations. This variation may result from larval nutrition or it may be indicative of the existence of subspecies. As well, there are still numerous questions regarding the attraction of *P. xylostella* and other crucifer-feeding insects to host plants.

A review by Renwick and Chew (1994) reported some confusion with regard to the role of glucosinolates in behavioral studies with *Pieris* spp. Alborn *et al.* (1985) pointed out that the search for host cues of cruciferous insects has been blurred by glucosinolates, and Schoonhoven (1976) stated the "idea of a single token stimulus [is] too simplistic". Published investigations of glucosinolates and isothiocyanates as oviposition stimuli for *P. xylostella*, often include phrases such as "...mustard oils [play a] ....possibly synergistic role..." (Pivnick *et al*, 1994), and "...leaf juice contains allyl isothiocyanate but is more stimulating to oviposition indicating that other factors contribute to the total effect." (Gupta and Thorsteinson, 1960).

Despite this, glucosinolates and isothiocyanates of crucifers have been, and continue to be, identified, characterized, and implicated as the defining characters for host choices by cruciferous insects such as *P. xylostella* (Table 7-I). Generally, studies that perpetuate this allegation do not define host choice (*e.g.* long-range searches or post-

alightment), and experimental designs lack attention to details (*e.g.* volatile studies that use extremely high concentrations to attract insects). Further, numerous studies of crucifer-feeding species have investigated the role of glucosinolates and/or isothiocyanates, and conclusions of these are sometimes extended to other insects rather than compared to them. Renwick *et al.* (1990) reported that cardenolides, which deter oviposition by *P. rapae*, do not have the same effect on *P. xylostella*, evidence that generalizations across cruciferous herbivores may be unrealistic.

Novel stimuli have been discovered recently in crucifers (*i.e.* chloroform fractions stimulate *P. xylostella* oviposition, Hughes *et al.*, 1997; 'CIF' stimulates tarsal receptors and oviposition by *Delia* spp., Roessingh *et al.*, 1992). Identification of these stimuli have not yet been reported, nor has the occurrence of these stimuli been recorded in plants other than the 'type species'. However, those studies clearly indicate that other compounds exist within the Cruciferae that are encountered and accepted by cruciferous insects. Of interest within the *Plutella* literature are several investigations that provided evidence for behaviorally repellent systems (Hough-Goldstein and Hahn, 1992; Dover, 1985) but those have not been explored further. As well, subtle clues regarding microstimuli that are evident in several publications (*e.g.* placement of eggs on outer leaves of *B. oleracea* is mainly adaxial, but abaxial on inner leaves (Talekar *et al.*, 1994)) appear to have been disregarded.

In truth, host plant identity is probably a composite of several stimuli that are combined in more than one modality and assimilated in the CNS. Harris and Miller (1982) reported synergistic effects of shape, color, and phytochemicals on *D. antiqua*

oviposition, and Städler and Buser (1984) reported a blend of compounds, unique to umbellifers, were necessary to elicit oviposition by the carrot fly, *Psila rosae*.

Activity of specific attractants (and repellents) within a modality likely depend on the search phase in question. Ramaswamy (1988) described oviposition behavior of oligophagous insects, including *P. xylostella*, as a composite of several phases and modalities in which flight, orientation, and landing involve vision and olfaction, host discrimination involves visual, olfactory, gustatory, and tactile stimuli, and oviposition involves specific odors and contact chemicals. That is, host patch searches may not involve the same stimuli as oviposition site determination within a patch, but these distinctions are not often made. For example, Palaniswamy *et al.* (1986) reported that volatiles of homogenized plants attracted moths in olfactometer choice assays and that these volatiles induced oviposition. However, that long-range volatiles would stimulate oviposition seems maladaptive because an oviposition stimulant should act at the site of oviposition rather than upwind of that site.

The most important conclusions of work described herein are summarized in the following statements.

1. Morphology of male and female reproductive systems exhibit some peculiar characteristics such as fused accessory glands in males and a simple bilobed colleterial gland in females. There is an apparent lack of typical lepidopteran spermatophore formation during copulation.

2. Morphometry of genitalia, possibly influenced by larval habitat, may effect isolation among populations, and suggests a need for further systematic study (to determine if this is truly a single species with a world-wide distribution).

3. *Plutella xylostella* performs four post-alighting behaviors during oviposition and these correlate with at least three modalities proposed by Ramaswamy (1988): antennal rotation (olfaction), antennation (gustation and possibly olfaction), ovipositor sweeping (gustation and tactile sensation), and egg deposition (tactile sensation).

4. Ovipositing females can distinguish between glossy and waxy *B. napus* and prefer to oviposit on glossy (whether genetic- or herbicide-induced), but this does not appear to correlate with stimulants that adhere to parafilm wax or to differences in headspace odors.

5. Perception of isothiocyanates by *P. xylostella* antennae is not uniform among isothiocyanates.

6. Host plant volatiles are perceived by male moths but to lesser extent than by females.

7. At least ten components of *B. napus* headspace extracts are perceived by females moths but none of these were identified as isothiocyanates. *Sinapis alba* headspace extracts differ from *B. napus* headspace extracts and three components unique to *S. alba* are perceived by *P. xylostella*. However, two extremely minor components of *B. napus* extracts (eluents H and I) elicited major EAG responses from *P. xylostella*, and one of these (eluent I) also occurs in *S. alba* extracts. The sensitivity to those components suggests that they are of some importance in *P. xylostella*-host interactions, but the level of host identification at which these and other volatiles may function remains unknown.

8. Volatiles of *D. carota* inhibit oviposition in small-scale bioassays.

9. Stimulatory compounds are not washed from leaves or leaf-treated parafilm with water only, but are washed from leaves with water after stripping waxes with dichloromethane and methanol. The nature of these compounds and what influences they may have on oviposition are unknown.

These conclusions, and the findings of others, suggest that *P. xylostella* uses isothiocyanates and other host plant volatiles for long-range attraction to host patches. These same volatiles may or may not be involved in short-range host discrimination; some volatiles (such as dimethyl trisulfide) may act exclusively in adult food source location. Further, short-range chemosphere components (*i.e.* with low volatility or occurring in very low concentrations) may be involved in host discrimination within a patch or in distinction between plant parts (*e.g.* young vs. old leaves). Non-volatile compounds at the leaf surface are likely responsible for detection of suitable egg depository sites. Glucosinolates may be involved but act, perhaps synergistically, with other components (*e.g.* non-polar components of Hughes *et al.*, 1997). Epicuticular waxes require further investigation; waxes amplify complexity of the leaf surface by providing variable tactile stimuli, increasing diversity of chemical makeup, and providing binding sites for chemical and biotic factors. Small changes in chemical and physical parameters at the leaf surface affect egg location, such as egg placement on the adaxial surface and along leaf veins.

Table 7-I. Overview of published data on chemical ecology of *Plutella xylostella* reproduction with respect to glucosinolates and isothiocyanates.

stimulus and/or assay	author/year
field observations of oviposition moths (descriptive)	Harcourt, 1957
preference for *B. oleracea* in choice assays of a single species from five plant families	Gupta and Thorsteinson, 1960
less eggs on sulfur deficient plants choice assays	Gupta and Thorsteinson, 1960
greater number of eggs with leaf juice choice assays of applications of mustard leaf juice and allyl isothiocyanate on artificial substrates	Gupta and Thorsteinson, 1960
egg production increased when females reared in presence of allyl isothiocyanate	Hillyer and Thorsteinson, 1969
egg production delayed females reared in absence of host plants	Hillyer and Thorsteinson, 1971
preference for *B. oleracea* in choice of five host types	Chand and Choudhary, 1977
oviposition decreased with application of rutin or coumarin to cabbage plants	Tabashnik, 1985
oviposition decreased with alcohol extracts of labiate herbs applied to cabbage leaf disks	Dover, 1985
number of eggs on brussel sprouts decreased when intercropped with clover	Dover, 1986
white mustard extracts most attractive in olfactometer assays; white mustard but not canola volatiles stimulated oviposition; EAGs exhibited to plant extracts	Palaniswamy *et al.*, 1986
oviposition increased with application of aqueous and anionic fractions of plant homogenate to filter paper; effect decreased when aqueous fraction treated with myrosinase or sulphatase	Reed *et al.*, 1989
ovarian development faster in lower pop'n density; greater number of viable eggs when mated with male exposed to host plants	Pivnick *et al.*, 1990a
attraction and oviposition activity in olfactometer assays with homogenized *B. juncea*	Pivnick *et al.*, 1990b
butanol extracts of *Erysimum cheiranthoides* applied to cabbage plants increased oviposition	Renwick *et al.*, 1990
extracts of *E. cheiranthoides*, *Tropaeolum majus*, and *Capsella bursa-pastoris* did not deter oviposition	Renwick and Radke 1990

Table 7-I. (continued)

stimulus and/or assay	author/year
aqueous extract of *Tanacetum vulgare* L. applied to cabbage leaf disks suppressed oviposition	Hough-Goldstein and Hahn, 1992
accelerated oocyte maturation in moths exposed to *B. juncea* seedlings	Pittendrigh and Pivnick, 1993
egg on cabbage mainly on outer leaves; eggs on outer leaves on upper surface, eggs on inner leaves on lower surface; number of eggs on chinese cabbage increased with trichome density.	Talekar *et al.*, 1994
attraction to *B. juncea* and *B. napus* seedlings in olfactometer; homogenized *B. juncea* attractive after myrosinase treatments; terpenes in active fraction may be stimulants	Pivnick *et al.*, 1994
greater number of eggs on artificial substrates with sinigrin or cabbage homogenate when *n*-alkanes applied than when *n*-alkanes not applied; no effect with *n*-alkanes only	Spencer, 1996
non-polar fractions of *B. oleracea* greatly stimulate oviposition	Hughes *et al.*, 1997

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APPENDICES

Appendix I: Solutions

I-1. Bouin's fixative

picric acid	75 ml
40% formaldehyde	25 ml
glacial acetic acid	5 ml

I-2. Mallory's triple stain (Barbosa, 1974)

Solution A

acid fuchsin (Allied Chemical)	0.5 g
deionized H_20	100 ml

Solution B

phosphomolybdic acid (Fisher Scientific)	1.0 g
deionized H_20	100 ml
orange G (Fisher Scientific)	2.0 g
aniline blue w.s. (Allied Chemical)	0.5 g

Combine phosphomolybdic acid and H_20. Add dyes. Filter before use.

I-3. Sinigrin for behavior observations and bioassays

10 mM sinigrin:

0.04155 g sinigrin (Sigma Chemical) in 10 ml deionized H_2O

0.5 mM sinigrin:

0.5 ml 10 mM sinigrin (above) and 9.5 ml deionized H_2O

I-4. EPTC working-solution

1 mM solution:

EPTC, 189.31 mw, 0.9546 d (Zeneca Agro)	89.25 µl
deionized H_2O	450 ml

serial dilutions to 10^{-6} M; stored at 4 °C until use.

Eight seedlings treated once/week or 3x/week with 20 ml of EPTC concentrations (10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M) for six weeks to determine optimal concentration for maximal reduction of waxes on healthy plants.

I-5. Physiological saline (pH = 6.5) for GC-EAG (E. Städler, unpubl.)

distilled H_2O	100 ml
glucose	6.380 g
KCl	0.048 g
KH_2PO_4	0.272 g
NaCl	0.070 g
$MgCl_2 \cdot 6\ H_2O$	0.244 g
$CaCl_2$	0.011 g
KOH	0.054 g

Stored at -12 °C.

I-6. Isothiocyanate standards for GC-EAG

1 mM solution:

allyl isothiocyanate, 99.15 mw, 1.02 d (Fluka)	0.10 ml
butyl isothiocyanate, 115.2 mw, 0.953 d (Fluka)	0.10 ml
phenyl isothiocyanate, 135.19 mw, 1.132 d (Fluka)	0.10 ml
hexane (Merck)	to 10.00 ml

0.1 mM solution:

1 ml of 1 mM standard solution (above) and 9 ml hexane.

Protected from UV and stored at -12 °C.

I-7. Terpene standards for GC-EAG

10 mM solution:

4-allylanisole, 148.21 mw, 0.965 d (Fluka)	15.36 µl
borneol, 154.25 mw (Fluka)	0.154 g
(+)-terpinen-4-ol, 154.25 mw, 0.933 d (Fluka)	16.50 µl
α-terpineol, 154.25 mw, 0.94 d (Merck)	16.41 µl
(-)-verbenone, 150.22 mw, 0.975 d (Fluka)	15.41 µl
hexane (Merck)	to 10.00 ml

Serial dilutions in hexane to 0.1 mM solution.

Stored at -12 °C.

I-8. *Plutella* Ringer's Solution (modified from Todd and Thornhill, 1989)

deionized H_2O	100 ml
Na_3 citrate	0.1176 g
$CaCl_2$	0.0588 g
KCl	0.2237 g
KH_2PO_4	0.1361 g
$MgSO_4$	0.4437 g
glucose	3.8010 g

osmolarity = 349; add 1 M KOH to raise pH to 7.15

Filtered with 0.22 µm Sterifil D-GS low extractable filter (Millipore, USA) and stored at -16 °C.

I-9. Tip-recording solutions

i. 50 mM KCl
0.3728 g KCl
100 ml deionized H_2O

ii. 100 mM KCl
0.7456 g KCl
100 ml deionized H_2O

iii. 250 mM KCl
1.864 g KCl
100 ml deionized H_2O

iv. 500 mM KCl
3.728 g KCl
100 ml deionized H_2O

v. 10 mM sinigrin + 100 mM KCl
0.0415 g sinigrin
0.07456 g KCl
10 ml deionized H_2O

vi. 10 mM sinigrin + 500 KCl
0.0415 g sinigrin
0.3728 g KCl
10 ml deionized H_2O

vii. 0.625 gle methanol extract + 100 mM KCl
0.07456 g KCl
10 ml methanol extract

viii. 0.625 gle methanol extract + 500 mM KCl
0.3728 g KCl
10 ml methanol extract

ix. 0.625 gle H_2O extract + 100 KCl
0.07456 g KCl
10 ml H_2O extract

x. 0.625 gle H_2O extract + 500 KCl
0.3728 g KCl
10 ml H_2O extract

xi. 0.3125 gle H_2O extract + 100 KCl
0.07456 g KCl
5 ml H_2O extract
5 ml H_2O deionized

xii. 1.25 gle H_2O extract + 100 KCl
0.07456 g KCl
20 ml H_2O extract reduced to 10 ml

xiii. 2.5 gle H_2O extract + 100 KCl
0.07456 g KCl
40 ml H_2O extract reduced to 10 ml

Molar concentrations of KCl refer only to amount added to extracts; absolute salt concentration in extracts before the addition of KCl was not determined.

Solutions xii and xiii were concentrated at 28 °C under vaccuum before the addition of KCl.

Appendix II: Histological protocols

II-1. Tissue fixation

Bouin's fixative	20 min under vaccuum (9 in/hg)
70% ethanol	overnight (scales removed in 70% ethanol)
80% ethanol	overnight
95% ethanol	2.5 hours
95% ethanol	2.5 hours
98% ethanol	3 hours
98% ethanol	3 hours
98% ethanol + CS_2	overnight
CS_2	8 hours
CS_2	8 hours
CS_2 + Paraplast plus®	3 days
infiltrate Paraplast plus®	15 minutes then 30 min under vaccuum (9 in/hg)

II-2. Mallory's triple stain

xylene	5 minutes
xylene	5 minutes
100% ethanol	3 minutes
100% ethanol	3 minutes
95% ethanol	3 minutes
80% ethanol	3 minutes
70% ethanol	3 minutes
super H20	3 minutes
Solution A	5 minutes
Solution B	15 minutes
super water	1 minute
95% ethanol	1 minute
95% ethanol	1 minute
100% ethanol	10 minutes
100% ethanol: xylene	4 minutes
xylene	5 minutes
xylene	2 minutes
mount in DPX	

Results: muscle and fibrin, red; nervous system, lilac; collagen, dark blue; mucus, connective tissue, and other hyaline substances, blue; chitin, red or blue according to its nature; yolk, yellow to orange.

Principle of method: Acid fuchsin is very soluble in water, particularly if slightly alkaline, as is tap water. Aniline blue w.s. rapidly dissolved in water and much more slowly in 90% ethanol. The phosphmolybdate intensifies the staining of fuchsin in some elements and helps decolorize the collagen. It increases the tendency for the collagen, rather than other elements, to stain with aniline blue w.s. In the mixture of acid dyes, Orange G and aniline blue w.s., each excludes the other from tissues which it selectively stains

Appendix III: Tissue preparations for scanning electron microscopy

III-1. Critical point drying

whole animals and excised parts sonicated in warm water with detergent	20 minutes
distilled H_20	rinse
transferred to 70% ethanol	overnight
held under vaccuum @ 8 in/hg	10 minutes
transferred to Bouin's fixative	10 minutes under vaccuum (8 in/hg)
vaccuum purged and repeated 5 times	
last repitition under vaccuum	35 minutes (9 in/hg)
transferred to 80% ethanol	overnight
transferred to 70% ethanol	overnight
placed in critical point drier chamber and filled with CO_2	5 minutes

Purged and repeated 5 times.
Last fill with CO_2 heated to 35 °C to force CO_2 through critical point.
Chamber purged.

Specimens used for antennal or tarsal sensilla surveys were not fixed in Bouin's fixative prior to critical point drying.
Aedeagi were not fixed in Bouin's fixative or critical point dried.

III-2. Cryopreparation of plant tissue

Portions of leaves were mounted on a brass stage (adaxial up or adaxial down), frozen in liquid N_2, and allowed to sublime under vaccuum. Immediately following sublimation, leaves were coated with gold using an Emitech K1250 preparation system and immediately transferred to a scanning electron microscope.

Appendix IV. Non-automated ANOVA Calculations

IV-1. Nested ANOVA calculations for 1995 (bold type) (1996 quantities are in square brackets) field studies (see Sokal and Rohlf, 1981)

Preliminary compuations:

1 - Grand total

$$= \Sigma^{a}\Sigma^{b}\Sigma^{c}\Sigma^{n}Y = \mathbf{657} \qquad [1489]$$

2 - sum of squared observations (samples)

$$= \Sigma^{a}\Sigma^{b}\Sigma^{c}\Sigma^{n}Y^{2} = 0^{2} + 1^{2} + 0^{2} + 0^{2} + \ldots.. 6^{2} + 9^{2} + 8^{2} = \mathbf{9295} \qquad [38569]$$

3 - sum of squared subsubgroup (type) totals divided by sample size of subsubgroups

$$= \frac{\Sigma^{a}\Sigma^{b}\Sigma^{c}(\Sigma^{n}Y)^{2}}{n} = \frac{1^{2} + 37^{2} + 2^{2} + \ldots.. 2^{2} + 30^{2}}{5} = \mathbf{6621.4} \qquad [31689]$$

4 - sum of squared subgroup (cage) totals divided by sample size of subgroups

$$= \frac{\Sigma^{a}\Sigma^{b}(\Sigma^{c}\Sigma^{n}Y)^{2}}{cn} = \frac{38^{2} + 20^{2} + 24^{2} + \ldots.. 45^{2} + 32^{2}}{(2 \times 5)} = \mathbf{4671.7} \qquad [23722.7]$$

5 - sum of squared group (stage) totals divided by sample size of groups

$$= \frac{\Sigma^{a}(\Sigma^{b}\Sigma^{c}\Sigma^{n}Y)^{2}}{bcn} = \frac{139^{2} + 292^{2} + 226^{2}}{(4 \times 2 \times 5)} = \mathbf{3891.525} \qquad [22308.1]$$

6 - correction term (*CT*) = grand total squared and divided by the totale sample size

$$= \frac{(\Sigma^{a}\Sigma^{b}\Sigma^{c}\Sigma^{n}Y)^{2}}{abcn} = \frac{657^{2}}{(3 \times 4 \times 2 \times 5)} = \mathbf{3597.075} \qquad [22171.21]$$

ANOVA Calculations:

7 - SS_{total} = $\sum\sum\sum\sum Y^2 - CT$ = quantity 2 - quantity 6 = **5697.925** **[16397.8]**

8 - SS_{groups} = $\frac{\sum(\sum\sum\sum Y)^2}{bcn} - CT$ = quantity 5 - quantity 6 = **294.45** **[136.89]**

9 - SS_{subgps} (subgps within grps) = $\frac{\sum\sum(\sum\sum Y)^2}{cn} - \frac{\sum(\sum\sum\sum Y)^2}{bcn}$ = quantity 4 - quantity 5 = **780.175** **[1414.6]**

10 - $SS_{subsubgps}$ (sbsbgps with sbgps) = $\frac{\sum\sum\sum(\sum Y)^2}{n} - \frac{\sum\sum(\sum\sum Y)^2}{cn}$ = quantity 3 - quantity 4 = **1949.7** **[7966.3]**

11 - SS_{within} (within subsubgrps; error) = $\sum\sum\sum\sum Y^2 - \frac{\sum\sum\sum(\sum Y)^2}{n}$ = quantity 2 - quantity 3 = **2673.6** **[6880]**

Degrees of freedom:

		1995	[1996]
Among groups (stages)	a - 1 =	3 - 1 = 2	[2 - 1 = 1]
Among subgroups (cages)	a(b-1) =	3 (4 - 1) = 9	[2 (5 - 1) = 8]
Among subsubgroups (types)	ab (c - 1) =	3 (4) (2 - 1) = 12	[2 (5) (2 - 1) = 10]
Within subsubgroups (samples)	abc (n - 1) =	3 (4) (2) (5 - 1) = 96	[2 (5) (2) (5 - 1) = 80]
Total	N - 1 =	120 - 1 = 119	[100 - 1 = 99]

Appendix V. GC-EAG System Optimization and Reference Checks

V-1. Volatile collection system contamination:

Airflow on as in collections but no plant material present. Elute traps and inject. Performed after 3-4 plant collections.

V-2. Collection system optimal time and flow rate:

Run collection system with chopped plants and measure peak heights from GC
Best collection at 0.5 l/min over 2 hours ✓
or 0.25 l/min over 3 hours.

V-3. "Breakthrough" of volatiles:

Two adsorbent traps set in-line; airflow at 0.5 l/min with plant material present;
second trap eluted after 1 hour, injected = no peaks;
" " 2 hours, injected = no peaks;
" " 3 hours, injected = no peaks;
" " 4 hours, injected = small peaks eluting.

V-4. Retention time of isothiocyanate standards:

Inject single standards in hexane and mixtures of standards; identify retention times with temperature program: 50°C for 2 min, 10°C/min to 80°C, 15°C/min to110°C for 5 min.

allyl isothiocyanate elutes at 334 seconds
butyl isothiocyanate elutes at 533 seconds
phenyl isothiocyanate elutes at 921 seconds

V-5. Optimal attenuation:

temperature program
40 °C 1 min; 5 °C/min to 100 °C; 10 °C/min to 240 °C for 10 min.
attenuation; 1 µl injections of 1 mM isothiocyanate standards
1, 4 delivers good peak shape but peaks overload
1, 8 delivers good peak shape but peaks overload
1, 16 delivers good peak shape but peaks overload
attenuation; 1 µl injections of 0.1 mM isothiocyanate standards
1, 4 delivers good peak shape but peaks overload ✓
1, 8 delivers good peak shape and height (~ 3/4 span of chart)
1, 16 delivers good peak shape only half the height of 1, 8.
attentuation; 1 µl injections of *B. napus* extracts
1, 4 many overloaded initially, moderate peaks eluting later ✓
1, 8 few peaks overloaded initially, moderate and small peaks later.

V-6. Elaboration of optimal GC temperature program for *Brassica* volatiles: preliminary runs with different programs to find sharp peaks with optimal separation

init °C	time	rate1	to °C	rate2	to °C	rate3	to °C	fin/ttl time
40	02	10	90	5	140	20	200	5 / 25 min
40	02	10	70	4	110	15	200	5 / 26 min
40	02	5	100	10	240			10 / 38 min
45	02	5	75	15	255			5 / 28 min
38	04	4	70	7	245			4 / 41 min
40	04	5	140	7	245			5 / 43 min
50	02	10	110	15	230			5 / 35 min
50	02	10	80	15	230			5 / 20 min ✓

V-7. Determination of volatile losses with reductive concentrating:

Inject.
Dry down with N_2 to ~ 100 µl. Inject.
Dry down with N_2 to ~ 20 µl. Inject.
Dilute with hexane to ~ 100 µl. Inject.
FID traces show losses with standards (including 0.1 mM trans-2-hexanal) are negligible; with *B.napus* glasshouse-grown glossy appear negligible.

V-8. Optimal air flow over antennae:

Trial and error; optimal flow determined by eye of signal-to-noise ratio with injection of standards (airflow too slow provides little or poor signal, airflow too fast causes increased noise levels).

Appendix VI. List of plant species and common names

VI-1. Order: Apiales

Family: Umbelliferae Jussieu (= Apiaceae Lindl.)
Daucus carota Linnaeus – carrot

VI-2. Order: Capparales

Family: Cruciferae Jussieu (= Brassicaceae Burnett)
Brassica juncea (Linnaeus) – oriental mustard
Brassica napus Linnaeus – Argentine rape, canola (and rutabaga)
Brassica oleracea Linnaeus – cabbage
Brassica oleracea var. *botrytis* – cauliflower
Brassica rapa Linnaeus – Polish rape, canola (and turnip)
Capsella bursa-pastoris (Linnaeus) – shepherd's purse
Erysimum cheiranthoides Linnaeus – wormseed mustard
Lepidium virginicum (unknown) – Virginia pepperweed
Rorippa indica (unknown) – indian marshcress
Sinapis alba Linnaeus – white mustard

VI-3. Order: Geraniales

Family: Tropaeolaceae Jussieu
Tropaeolum majus Linnaeus – garden nasturtium

VI-4. Order: Asterales

Family: Asteraceae Dumort (= Compositae Giseke)
Tanacetum vulgare Linnaeus – tansy

Appendix VII. List of insect species and common names

VII-1. Order: Homoptera

Family: Aphididae
Brevicoryne brassicae (Linnaeus) – cabbage aphid

VII-2. Order: Coleoptera

Family: Chrysolmelidae
Leptinotarsa decemlineata Say – Colorado potato beetle
Phyllotreta cruciferae (Goeze) – crucifer flea beetle

Family: Curculionidae
Ceutorhynchus assimilis Paykull – cabbage seed weevil

VII-3. Order: Diptera

Family: Tephritidae
Dacus oleae (Gmelin) – olive fruit fly

Family: Psilidae
Psila rosae (Fabricius) – carrot rust fly

Family: Anthomyiidae
Delia antiqua (Meigen) – onion maggot
Delia floralis (Fallén) – turnip maggot
Delia radicum (Linnaeus)
(=*D. brassicae* (Wiedemann)) – cabbage root maggot

VII-4. Order: Lepidoptera

Family: Hepialidae
Hepialis humuli (Linnaeus)

Family: Tortricidae
Adoxophyes orana Walsingham – apple moth
Choristoneura fumiferana (Clemens) – spruce budworm
Cydia (=*Laspeyresia*) *pomonella* (Linnaeus) – codling moth
Cydia nigricana (Fabricius) – pea moth
Grapholitha molesta (Busck) – oriental fruit moth
Laspeyresia caryana (Fitch)

Family: Yponomeutidae
Yponomeuta cagnagellus
Yponomeuta vigintipunctatus

Family: Plutellidae
Acrolepia (=*Acrolepiopsis*) *assectella* Zeller – leek moth
Plutella xylostella (Linnaeus)
(=*P. maculipennis* (Curtis)) – diamondback moth

Family: Pyralidae
Chilo partellus (Swinhoe) – maize stem borer
Diatraea grandiosella Dyar – southwestern corn borer
Diatraea saccharalis (Fabricius) – sugarcane borer
Ephestia (=*Anagasta*) *kühniella* Zeller – mediterranean flour moth
Homeosoma nebulella Den. & Schiff – European sunflower moth
Ostrinia nubilalis (Hübner) – European corn borer
Plodia interpunctella (Hübner) – Indianmeal moth

Family: Papilionidae
Papilio polyxenes (Fabricius) – black swallowtail

Family: Pieridae
Pieris brassicae (Linnaeus) – large white butterfly
Pieris rapae (Linnaeus) – cabbage butterfly

Family: Nymphalidae
Euphydryas editha Boisduval – Edith's checkerspot

Family: Danaidae
Danaus plexippus (Linnaeus) – monarch butterfly

Family: Bombycidae
Bombyx mori (Linnaeus) – silkworm

Family: Saturniidae
Antherea polyphemus (Cramer) – polyphemus moth

Family: Noctuidae
Agrotis segetum Schiff – turnip moth
Heliothis (=*Helicoverpa*) *zea* (Boddie) – corn earworm
Helicoverpa armigera Linnaeus
Helicoverpa assulta Linnaeus
Mamestra configurata Walker – bertha armyworm
Pseudaletia unipuncta (Haworth) – armyworm
Peridroma margaritosa (Haworth)

Spodoptera frugiperda (J.E.Smith) – fall armyworm
Trichoplusia ni (Hübner) – cabbage looper

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